CANADIAN JOURNAL OF RESEARCH

VOLUME 21 MARCH, 1943 NUMBER 3

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NATIONAL RESEARCH COUNCIL
OTTAWA, CANADA

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	Annual	*Single Copy*
A and B	$ 2.50	$ 0.50
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The Canadian Journal of Research is published by the National Research Council of Canada under authority of the Chairman of the Committee of the Privy Council on Scientific and Industrial Research. All correspondence should be addressed:

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Canadian Journal of Research

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

VOL. 21, SEC. C. MARCH, 1943 NUMBER 3

A STUDY OF SOME FACTORS AFFECTING THE PATHOGENICITY OF *VERTICILLIUM ALBO-ATRUM* R. & B.[1]

BY COLIN D. MCKEEN[2]

Abstract

An epidemic of Verticillium wilt, which occurred in the Niagara Peninsula in 1940, involved many hosts, from seven of which the fungus was isolated; the isolates were compared with respect to cultural characters and pathogenicity. Although the isolates varied slightly in morphology and pathogenicity, they are all referred to *Verticillium albo-atrum* R. & B.

A considerable residuum of inoculum was demonstrated in the soil in May, 1941, following the 1940 epidemic. The optimum temperature for vegetative growth of the fungus is about 24° C. which closely approximates the optimum for disease incidence. Wilt symptoms appear slightly sooner and the temperature range of heavy disease incidence is broader (18° to 29° C.) at high than at optimum soil moisture (21° to 27° C.). The fungus persisted and was equally aggressive after three months in fine sandy loam, medium clay loam, and red clay loam under cropped and fallow conditions, except when the soil was fallow and dry during the period. Growing a susceptible host in infested soil for three months did not influence the activity of *Verticillium* more than did an immune host. Inoculum in a resting condition must be incorporated in moist soil a few days before it can readily infect plants. The addition of green plant residues and two organic acids caused a slight suppression of fungous activity.

Air and soil temperatures and precipitation for May, June, July, and August for 1939, 1940, 1941, and 1942 have been examined. It was found that soil temperatures high enough for disease development are not likely to be encountered before late June and then the disease developed seriously only when the soil moisture was uniformly high during the months of May, June, and July. The relative infrequency of serious outbreaks of Verticillium wilt on the Niagara Peninsula thus appears to be due to the low soil moisture that ordinarily pertains during that part of the growing season when soil temperatures are high enough to favour the fungus.

Introduction

The epidemiology of Verticillium hadromycosis on the Niagara Peninsula presents many interesting problems, the solution of which would add much to our knowledge of the bionomics of soil-borne parasites. Characteristically there occurs, about one year in six, an outbreak of epiphytotic proportions involving a large number of hosts, including fruit trees, small fruits, vegetables, and ornamentals. This is in striking contrast to the occurrence of other soil-borne parasites in the same area, in so far as they tend to recur year after year, with considerable variation in disease incidence from season to season

[1] *Manuscript received November 18, 1942.*

Contribution from the Department of Botany, University of Toronto, Toronto, Ont. Based on a thesis presented in May, 1942, to the University of Toronto in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

[2] *Assistant, Department of Botany, University of Toronto.*

and from locality to locality in any given year. This suggests that, in the case of *Verticillium*, some factor of the environmental complex may operate to the exclusion of the others in limiting infection, or the expression of characteristic disease symptoms, or that infection occurs only under certain concatenations of environmental factors which are only infrequently encountered in this district. In the hope of shedding some light on the cause of the erratic occurrence of this disease a study was undertaken of the influence of various factors and of various combinations of factors on the parasitic activity of the *Verticillium* fungus.

Isolation of the Fungus

The summer of 1940 was a season in which Verticillium wilt was extremely common throughout the Niagara Peninsula and a large number of hosts manifested typical symptoms. Large numbers of the following plants were infected: young peach trees, barberry, roses, raspberry, potato, tomato, muskmelon, and eggplant. In one nursery containing barberry and roses 50% of the shrubs were infected and many of these were killed. Bewley (1), Van der Meer (6), Rudolph (5), and others have described in detail the symptoms on most of the hosts reported, so that they will be omitted here.

Verticillium was isolated from diseased barberry, peach, rose, potato, eggplant, muskmelon, and chrysanthemum of which chrysanthemum only had been grown indoors.

Comparison of Isolates

When the isolates obtained from the seven hosts were studied as to their cultural characteristics on potato dextrose agar in Petri plates, it was evident that there were two distinct types of vegetative growth. The isolate obtained from chrysanthemum produced a profuse growth of white aerial mycelium which in old cultures was underlain by a black film composed of dark, thick-walled, septate hyphae and a few scattered "microsclerotia", some of which were submerged. The other six isolates produced variable but much smaller amounts of aerial mycelium very little of which was white and most of which was grayish-black. In addition, on the surface of the agar there developed a black crust which consisted of numerous, dark, thick-walled, septate chlamydospore-like hyphae and of numerous "microsclerotia". The isolates of this latter type that were obtained from eggplant and muskmelon produced more white aerial mycelium and a thinner black crust on the agar surface than did those obtained from barberry, peach, rose, and potato.

The writer thought it desirable to test the pathogenicity of the seven *Verticillium* isolates on various hosts. To simulate natural conditions of infection as closely as possible seeds were sown in soil infested with a sand–cornmeal–Verticillium mixture. The following seeds were sown: African marigold (*Tagetes erecta* L.), larkspur (*Delphinium Ajacis* L.), California poppy (*Eschscholtzia californica* Cham.), common foxglove (*Digitalis purpurea* L.), snapdragon (*Antirrhinum majus* L.), *Godetia azaleiflora*, sweet pea (*Lathyrus odoratus* L.), annual phlox (*Phlox Drummondii* Hook.), China aster (*Calli-*

stephus chinensis Nees), stock (*Matthiola incana* R. Br.), bachelor's button (*Centaurea Cyanus* L.), and tomato (*Lycopersicon esculentum* Mill. var. *vetomold*). Thirteen weeks after sowing the seeds the symptoms of Verticillium hadromycosis could not be recognized in China aster, stock, larkspur, and California poppy, but were apparent in African marigold, bachelor's button, Godetia, snapdragon, sweet pea, phlox, and tomatoes. The symptoms varied to a considerable extent with the different plants and some hosts exhibited a differential reaction to the various isolates.

The *Verticillium* isolated from chrysanthemum was more virulent than the other isolates on both snapdragon and bachelor's button, but was the least virulent on tomato and sweet pea. The other six isolates displayed approximately the same degree of pathogenicity on all of the hosts. Although the extremes are relatively easily distinguished, the isolates as a whole form a gradually intergrading series, consequently the writer prefers to regard them all as the one species, *V. albo-atrum* R. & B.

Temperature Relations of the Fungus in Culture

As a prelude to a study of the influence of environmental factors on disease development, the effect of temperature upon the growth rate of *Verticillium* in culture was studied. In this experiment the strains isolated from chrysanthemum and from potato were used and their growth rates were studied on cornmeal agar and potato dextrose agar. Each Petri plate was inoculated by placing a bit of mycelium on the surface of the agar in the centre of the plate. The growth rate was studied at 5°, 7.5°, 12°, 16°, 20°, 22°, 24°, 28°, 32°, and 35° C. from three plates that were subjected to each temperature. Every 48 hr. the diameter of the colony was measured. Final observations were made at the end of 12 days, at which time the plates that showed no obvious growth were transferred to an oven at 22° C. for one week.

In Fig. 1 the colony diameter of both isolates on potato dextrose agar and on cornmeal agar at the end of 12 days is plotted against temperature. Growth occurred at all temperatures except at 5°, 32°, and 35° C. After these plates had been transferred to an oven at 22° C. and left for one week growth took place only in those that previously had been incubated at 5° C. The minimum temperature for growth was slightly below 8° C. and the maximum temperature tolerated by the fungus was between 28° and 32° C. The greatest amount of growth was made over a range from 20° to 28° C.; the optimum growth temperature was 24° C. except for the isolate from potato on potato dextrose agar in which the optimum was at 22° C. The growth rate of the isolate from chrysanthemum was slightly greater than that from potato.

Epidemiology

The Influence of Soil Temperature and Soil Moisture on Disease Incidence

The influence of soil temperature and soil moisture on disease incidence in young tomato plants was investigated by using Wisconsin soil temperature

tanks in the University of Toronto greenhouses. The atmospheric humidity varied from about 45 to 70% while the air temperature remained at about 21° C.

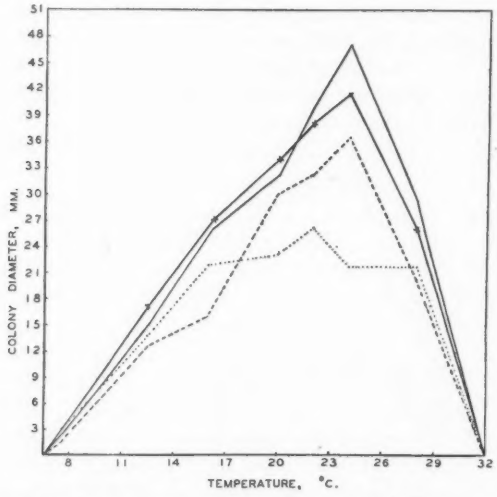


FIG. 1. *The effect of temperature on the growth rate of Verticillium from potato and chrysanthemum on potato dextrose agar and cornmeal agar;* × —— × = *isolate from chrysanthemum on potato dextrose agar;* . . . . . = *isolate from potato on potato dextrose agar;* ——— = *isolate from chrysanthemum on cornmeal agar;* - - - - - = *isolate from potato on cornmeal agar.*

The soil consisted of a mixture of two parts clay loam, one part humus, and one part sand. It was sterilized by steam and two days later was infested with *Verticillium*. The fungus which was used in the remainder of the experiments reported in this paper produced "microsclerotia" abundantly and was originally isolated from potato. The inoculum consisted of a sand–cornmeal mixture on which *Verticillium* had been growing 12 days. The degree of infestation of the soil was approximately 4% by volume. The moisture content of the soil at the time of infestation was slightly below optimum.

Each can in the soil temperature tanks was prepared as follows. A 4 in. layer of sterilized soil was placed in the bottom of the can. An inverted flower pot was placed above this layer and a glass tube with holes one inch apart in the sides led from the pot to three inches above the surface of the soil in the can. The infested soil was added to the can around the glass tube. Water, when required, was added through the glass tube permitting the moisture to spread uniformly through the soil in the upper regions of the can.

Six cans were prepared after this fashion for each of the six temperature tanks. A check series was set up employing two cans at each temperature filled with a similar soil except that the fungus was not present in the sand–cornmeal mixture. After the cans had been set up they were left at room

temperature of 21° C. for three days to allow for uniform growth and spread of mycelium. On the fourth day the cans were placed in the temperature tank cans for 24 hr. before the tomatoes were transplanted into them in order that the soils might reach the required temperatures. The tanks were maintained at temperatures of 12°, 16°, 20°, 24°, 28°, and 32° C.

Eight tomato plants about 4 in. tall, uniform in size, and possessing the same number of leaves were transplanted into each can. They were situated around the inside margin of the can so that they were all equidistant from the glass tube in the centre. These tomatoes had grown in sterilized soil and most of the soil at the time of transplanting was easily shaken from the roots before the small plants were placed in the tank cans. After transplanting, sufficient water was added to four cans in each tank to obtain a high soil moisture and to the remaining four cans to obtain an optimum soil moisture. The moisture content determined at the end of the experiment from the top two inches of soil was 63% of the moisture holding capacity for those cans at high soil moisture and 45% for those at optimum soil moisture. Moisture levels were maintained during the course of the experiment by periodic weighings of the cans and by the addition of water to return to original weight. A quarter inch layer of sterile sand was added to the soil in each can to establish a uniform surface for evaporation.

Daily records of the number of plants that showed wilt symptoms in each can were taken until the 27th day after transplanting. At that time the extent of wilting of each plant was noted and the amount of stunting was determined by the difference in height and weight of the plants in the infested and non-infested soils at each temperature and soil moisture.

The final analysis of the experiment is shown in Fig. 7 in which disease rating is plotted against temperature. The disease rating, which was based on stunting and wilting of leaves, was determined according to the following formula:

$$\tfrac{1}{2}\left[\left(\frac{\text{Av. height of checks minus av. height of infected plants}}{\text{Av. height of checks}} \times 100\right)\right.$$
$$\left. + \left(\frac{\text{Av. weight of checks minus av. weight of infected plants}}{\text{Av. weight of checks}} \times 100\right)\right]$$
$$\times \text{ (Average number of leaves wilting per plant).}$$

Inspection of Fig. 2 shows that the optimum disease incidence at high soil moisture was limited to the range of 20° to 28° C. and that very little wilt occurred at 12° and 16° C. At optimum soil moisture, wilt again occurred from 16° to 28° C. with a conspicuous peak, however, at 24° C., and the curve for disease incidence is not so broad as at the higher moisture. In general, it would appear that infection may occur throughout the temperature range of from 12° to 32° C. but that a soil temperature of from 20° C. to 28° C. is most conducive to disease expression.

On comparing the effect of temperature upon disease expression with its effect upon the growth of the fungus in culture it can be seen that the temperature range that is favourable for disease is also one in which the fungus makes

a good vegetative growth. Since the optimum temperature for disease incidence and the optimum for vegetative growth of *Verticillium* coincide it would appear that this temperature factor influences the degree of disease incidence primarily through its effect upon the fungus. It was noted that there was a small amount of wilt at 32° C. in the temperature tank series but no growth occurred in culture at this temperature. It was discovered, however, that the temperatures varied from 30° to 31° C. in the upper inch of soil in each can of this tank. In a later cultural test it was found that *Verticillium* made a small amount of growth at these temperatures.

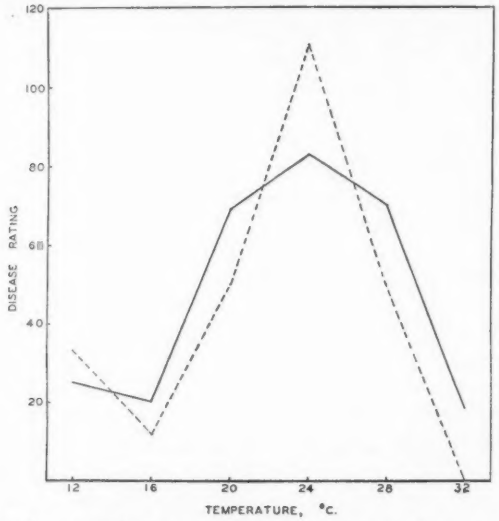


FIG. 2. *The effect of soil temperature and soil moisture on the amount of wilting of tomatoes;* ——— = *high soil moisture;* - - - - - = *optimum soil moisture.*

The Persistence of Verticillium in Naturally Infested Soil Incubated at Various Temperatures

A soil temperature tank experiment was set up using a light sandy loam soil obtained early in May, 1941, from a nursery plot in which barberry had been heavily infected the previous summer. Tomato plants about 4 in. high were transplanted into the cans. Three cans in each tank were held at high soil moisture, three at optimum soil moisture, and one at low soil moisture. The moisture was maintained by weighing the cans periodically and adding the required amount of water. The tanks were maintained at temperatures of 12°, 16°, 20°, 24°, 29°, and 33° C. The atmospheric humidity showed a considerable fluctuation varying from 20% on a hot day to 70% at night. Likewise the air temperature varied from 70° to 90° F. and on several hot days the temperature reached 100° F. Daily observations were taken of the number of plants wilting.

At the end of 25 days none of the tomatoes in any of the cans had wilted. A number of the plants, however, possessed yellowed basal leaves and some plants had dropped one or two of the lowest leaves. This yellowing of the lowermost leaves was rather pronounced at 12°, 16°, 20°, and 24° C., but was only slight at 29° C. and was absent at 33° C. This was thought initially to have been purely an effect of temperature upon the host. Bewley (1) states that under conditions least favourable to the fungus, i.e. at temperatures above 25° C., the leaves do not wilt but gradually desiccate from the base of the plant upwards. Finally death ensues. Later in the summer of 1941, particularly in July and early August, it was found that tomato plants growing in heavily infested soil usually did not wilt, but instead the leaves yellowed progressively from the base to the top of the plant. Since the writer was not sufficiently acquainted with these yellowing symptoms, the disease incidence at the various soil moistures and soil temperatures could not be assessed at this time.

Because the results of this experiment were inconclusive this experimental set-up was continued from June 20 to September 12 with a few modifications. The cans previously maintained at high soil moisture were brought to the optimum level. In three cans in each temperature tank tomatoes were allowed to grow while the tomato plants were removed from the other three cans and the soil was left fallow for the summer. Unsterilized clay loam soil was infested with 2% of its volume of a 14-day-old sand–cornmeal–Verticillium inoculum and was added to the remaining two cans in each tank. Seven tomato plants were transplanted into one of these two cans in each tank while the soil in the other was left fallow. Optimum soil moisture was maintained in this latter series. The surface of the soil in every can was covered with a quarter-inch layer of sand above which was placed a pad of non-absorbent cotton to maintain the upper inch of soil in each can at the same temperature as the water in the tank.

On September 12, the tomatoes were removed from the cans in which they had been growing. All the temperature tanks were adjusted to 23° C. and two days later seven tomato plants that had been growing in sterilized soil were transplanted into each can. In transplanting, care was taken not to transport soil from one can to another on the hands or transplanting tools. The soil moisture was maintained at an optimum level. Daily records were obtained of the number of plants wilting at each temperature. Final observations were taken seven weeks after transplanting.

The survival of *Verticillium* under these conditions is shown in Table I. It is apparent that *Verticillium* persisted in all of these soils except when the soil had been held at 33° C. The presence of this host had no apparent influence upon the persistence of the fungus.

Table II shows the progressive wilting of tomato plants growing in artificially infested soil. The fungus did not survive at 33° C. but did persist at all other temperatures. From these results it seems that the presence of a host over a three month period does not influence the activity of *Verticillium*

to any obvious extent. Since a small number of plants were used in this experiment and a number of these were killed by *Phytophthora*, it would be desirable to conduct a similar experiment with larger numbers and extending over a longer period of time.

TABLE I

THE EFFECT OF A THREE MONTH INCUBATION OF NATURALLY INFESTED SOIL AT DIFFERENT TEMPERATURES WITH AND WITHOUT A HOST

Temperatures at which soils were held for a three month period	Host present	Fallow
12° C.	6/20*	6/18
16° C.	5/21	7/18
20° C.	8/21	4/21
24° C.	2/21	5/20
29° C.	2/21	5/21
33° C.	0/21	0/21

** The numerator refers to the number of plants wilted. The denominator refers to the total number of plants in the given series.*

TABLE II

THE EFFECT OF A THREE MONTH INCUBATION AT VARIOUS TEMPERATURES ON THE PERSISTENCE OF *Verticillium* IN ARTIFICIALLY INFESTED UNSTERILIZED SOIL WITH AND WITHOUT A HOST

No. of days after transplanting	Number of tomato plants, out of seven, wilting in soil held for three months at:											
	12° C.		16° C.		20° C.		24° C.		29° C.		33° C.	
	Fallow	With host	Fallow	With host	Fallow	With host	Fallow	With host	Fallow	With host	Fallow	With host
16	0	0	0	*(2) 0	0	0	0	(3) 0	0	(4) 0	0	0
19	0	0	0	1	0	0	0	1	0	0	0	0
21	1	2	1	2	1	2	1	1	0	1	0	0
23	2	4	3	2	3	3	1	1	0	2	0	0
26	3	6	5	4	5	4	1	2	1	2	0	0
28	5	7	6	5	7	5	1	4	1	2	0	0
33	7	7	7	5	7	6	6	4	1	3	0	0
37	7	7	7	5	7	7	6	4	3	3	0	0
42	7	7	7	5	7	7	7	4	7	3	0	0
53	7	7	7	5	7	7	7	4	7	3	0	0

** The figures in parentheses represent the number of plants that were killed with Phytophthora parasitica owing to a contamination.*

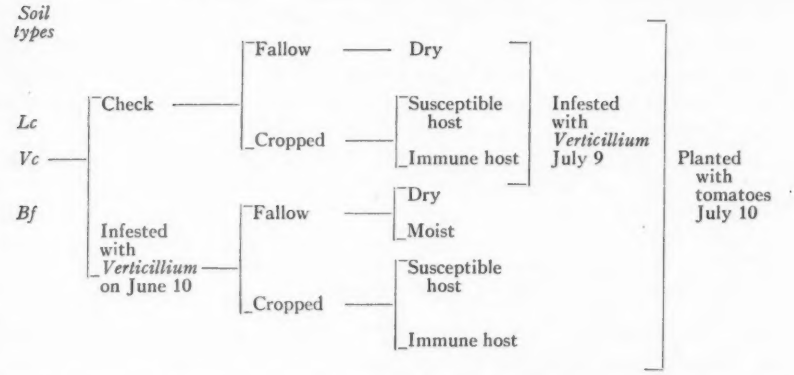
From Table II it can be seen that 100% of the plants that were transplanted into soils artificially infested and previously incubated at 12°, 16°, 20°, 24°, and 29° C. for three months had wilted at the end of seven weeks. From Table I not more than 36% of the plants, which had been transplanted into naturally infested soils after a three month incubation period at the various temperatures, became infected. This indicates the presence and the level of activity of *Verticillium* in natural soils one year after a disease outbreak. From this experiment it is evident that there is considerable residual inoculum held over in the soil after a winter season. If this is a typical occurrence an epiphytotic might be expected in the year following an outbreak provided that favourable conditions for disease development are present.

The Persistence and Aggressiveness of Verticillium in Different Types of Soil under Cropped and Fallow Conditions

This experiment was conducted in the greenhouses at the Vineland Experiment Station in the summer of 1941. On June 10, about two cubic feet of each of three different soils were infested uniformly with 3% of its volume of a 20-day–old, sand–cornmeal–Verticillium inoculum. The soils were Lockport clay loam (*Lc*), Vineland clay loam (*Vc*), and Vineland fine sandy loam (*Bf*). The Lockport clay loam (*Lc*) is a poorly drained red clay underlain by greenish-gray shale and red clay, low in organic matter, with a pH of 5.5 to 6.2. The Vineland clay loam (*Vc*) is a poorly drained clay dotted with sand knolls, high in organic matter with a pH of 6.2 to 7. The Vineland fine sandy loam (*Bf*) is an imperfectly drained brown sandy loam underlain with clay, fair in organic matter, with a pH varying from 6.2 to 6.8. After infestation the soils were left in flats on the greenhouse bench under various conditions. One half of each type of soil was left in a fallow condition, while the remainder was under crop. Of the fallow portion of each type of soil, one half was left dry, while the other portion was kept moist by watering copiously every morning. Small tomato plants, as a susceptible host, were transplanted into one half of the cropped portion and buckwheat, as an immune host, was sown in the other half. As a check series in this experiment a quantity of each of these soils, which was not infested, was divided into three portions; one was left dry and fallow, a second was sown with buckwheat, and into the third tomatoes were transplanted.

A schematic representation of the treatment of the soils in this experiment up to the time of the first test is shown below.

On July 10, the first experiment was set up to determine the level of aggressiveness and persistence of *Verticillium* under these various conditions. Gallon-sized crocks were filled with each type of soil that had been infested on June 10. The soils in the check series were infested on July 9 with the usual inoculum at the same rate by volume and then were placed in crocks. Seven tomato plants about 3 in. tall were transplanted into all the crocks of soil. On each of the first three days after transplanting 150 cc. of Pfeffer's (modified) nutrient solution were added to each crock of soil and subsequently



water was added every morning. The temperature of the greenhouse showed considerable fluctuation, remaining at about 70° to 75° F. during the night, but rising to 100° F. every afternoon with occasionally a temperature of 114° F. being recorded. The humidity also showed considerable variation, fluctuating between 15 and 60% every 24 hr. Daily observations were taken on the number of plants wilting in each crock.

In the early part of this experiment it was apparent that some plants would manifest typical wilt symptoms on a given day and probably would not show them again for several days. Although a large percentage of the plants did not actually wilt, at the end of 39 days all showed yellowing of the two lowest leaves and these leaves had fallen from some plants. All the plants in this test were heavily infected with *Cladosporium fulvum* and those growing in Lockport clay loam and Vineland fine sandy loam were badly stunted. Because unfamiliar atypical symptoms were expressed in many plants a satisfactory assessment of aggressiveness of *Verticillium* could not be made at this stage.

On August 13, the second test of the persistence and level of aggressiveness of *Verticillium* in these same soils was begun. The procedure was similar to that outlined above except that the soils in the check series were infested on August 12. Every day for the first week after transplanting the tomatoes 150 cc. of nutrient solution were given to each crock of soil and water was substituted for the remainder of the experiment. During this test the temperature of the greenhouse remained between 70° and 85° F. except twice when it rose to 95° F. The atmospheric humidity varied from 20 to 70%.

Table III shows the progressive wilting of tomatoes in the various soils and this is also shown in Fig. 3 in which the percentage of plants diseased is plotted against time. In constructing the curves the total numbers of plants wilting in the three soil types in each particular series were combined to give the per cent wilting each day. In this test the total number of plants wilting at the end of 34 days in each particular group was not appreciably different except for those growing in fallowed soil which was left dry for two months

TABLE III

THE EFFECT OF FALLOWING AND CROPPING FOR TWO MONTHS UPON THE PERSISTENCE AND AGGRESSIVENESS OF *Verticillium*, IN LOCKPORT CLAY LOAM (*Lc*), VINELAND CLAY LOAM (*Vc*), AND VINELAND FINE SANDY LOAM (*Bf*)

Number of days after transplanting (Aug. 13)	Left dry; infested Aug. 12			Cropped with tomato; infested Aug. 12			Cropped with buckwheat; infested Aug. 12			Cropped with tomato; infested June 10			Cropped with buckwheat; infested June 10			Fallow; left dry; infested June 10			Fallow; kept moist; infested June 10		
	Bf	*Lc*	*Vc*	*Bf*	*Lc*	*Vc*	*Bf*	*Lc*	*Vc*	*Bf*	*Lc*	*Vc*	*Bf*	*Lc*	*Vc*	*Bf*	*Lc*	*Vc*	*Bf*	*Lc*	*Vc*
10	0	2*	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12	0	2	0	0	0	0	2	1	0	0	0	1	0	1	1	0	0	0	0	0	1
13	0	2	2	2	0	0	2	1	1	3	2	3	2	1	1	0	1	1	3	0	1
14	1	2	2	4	0	1	3	2	1	5	2	4	4	4	3	0	1	1	3	1	1
17	2	2	3	4	1	1	3	2	1	6	4	4	5	5	3	0	1	1	6	2	3
21	2	3	6	7	2	3	4	5	3	7	5	5	6	5	4	0	1	1	7	5	5
23	4	3	6	7	5	4	5	5	3	7	5	5	6	5	4	0	1	1	7	5	7
26	5	3	6	7	6	5	7	5	4	7	6	6	6	6	5	0	2	4	7	5	7
32	5	6	7	7	7	7	7	5	6	7	7	6	6	6	7	0	6	6	7	7	7
34	5	7	7	7	7	7	7	5	6	7	7	6	6	6	7	2	6	7	7	7	7

* *Number of tomato plants, out of seven, wilted on a given day.*

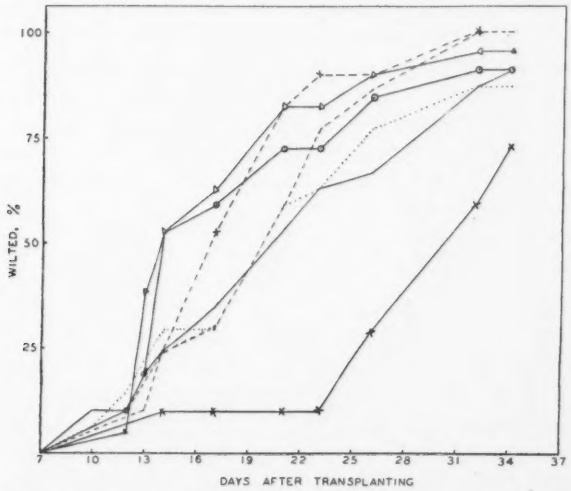


FIG. 3. *The rate of wilting of tomatoes growing in soils infested with V. albo-atrum and incubated for two months under various fallowed and cropped conditions;* × —— × = *infested June 10, left dry;* × - - - - - × = *infested June 10, kept moist;* ⊙ —— ⊙ = *infested June 10, cropped with tomato;* - - - - - = *infested Aug. 12, cropped with tomato;* △ ——— △ = *infested June 10, cropped with buckwheat;* . . . . . = *infested Aug. 12, cropped with buckwheat;* ——— = *infested Aug. 12, no previous crop.*

after being infested. The total number of plants wilted and the rate of wilting in the three types of soil in any group was approximately the same, except for those growing in the Vineland fine sandy loam soil which had been left dry. From Fig. 3 it can be seen that with one exception the curves assume approximately one form. The exception represents the rate of wilting of plants where the soils had been infested for two months and left in a dry fallow condition. Since this curve rises rather steeply after the tomatoes have been in the soils for 22 days it would appear that the fungus has persisted during this dry period but has not been in an active parasitic state at the time the test was begun. The rate of wilting was slightly lower in those plants that were growing in recently infested soil. This might be explained by the fungus not having sufficient time to invade the soil thoroughly before the tomatoes were transplanted into it.

On September 6, the third test of the persistence of *Verticillium* under these conditions was begun. The procedure was almost identical with that outlined for the second test. In the check series the soils were infested on September 5 and also a control was inserted in which the soils were not infested. A nutrient solution was given to the tomatoes for the first week after transplanting. Observations were taken for the next 21 days and the final one was taken at the end of 31 days. During this experiment the air temperature rose to 90° F. for the first three days after transplanting but remained between 70° and 86° F. for the remainder of the experiment. Once again the atmospheric humidity fluctuated between 20 and 70%.

Table IV shows the progressive wilting of tomatoes transplanted into the various soils. From the table it is evident that the fungus persisted in Lockport clay loam, Vineland clay loam, and in Vineland fine sandy loam soils both under cropped and fallow conditions for a three month period. Once again approximately the same number of plants became infected in these three types in each series except in the infested Vineland fine sandy loam which was left dry and fallow. Only two out of seven plants became infected in this soil whereas five out of seven became infested in both Lockport clay loam and Vineland clay loam soils. This loss of fungus activity in this soil might be due to the exposure to a higher temperature for June and July. The fallow soils that were left dry were contained in flats stacked in tiers on the greenhouse bench with the Vineland fine sandy loam soil in the upper flat. The temperature recorded in this flat in the upper inch of soil on July 29, a hot afternoon, was 36° C., whereas the temperature of the soil in the flats below was 29° C.

From the table it is apparent that there is a distinct lag in the onset of wilting in those tomatoes transplanted into the infested soil that had been left fallow and dry and even after 30 days only 58% of the plants wilted. The development of wilt in the fallow soils that were kept moist was in striking

TABLE IV

THE EFFECT OF FALLOWING AND CROPPING FOR THREE MONTHS UPON THE PERSISTENCE AND AGGRESSIVENESS OF *Verticillium* IN LOCKPORT CLAY LOAM (*Lc*), VINELAND CLAY LOAM (*Vc*), AND VINELAND FINE SANDY LOAM (*Bf*)

Number of days after transplanting (Sept. 6)	Left dry; infested Sept. 5			Cropped with tomato; infested Sept. 5			Cropped with buckwheat; infested Sept. 5			Cropped with tomato; infested June 10			Cropped with buckwheat; infested June 10			Fallow; left dry; infested June 10			Fallow; kept moist; infested June 10		
	Bf	*Lc*	*Vc*	*Bf*	*Lc*	*Vc*	*Bf*	*Lc*	*Vc*	*Bf*	*Lc*	*Vc*	*Bf*	*Lc*	*Vc*	*Bf*	*Lc*	*Vc*	*Bf*	*Lc*	*Vc*
8	0	1*	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9	0	3	1	1	4	0	1	3	1	0	0	0	0	1	0	0	0	0	1	0	0
10	1	3	3	1	4	2	3	4	1	0	1	1	1	1	0	0	0	0	3	3	0
11	1	4	4	1	7	2	4	4	3	0	2	2	2	1	1	0	0	1	5	3	1
13	2	4	4	2	7	2	4	6	5	0	2	3	2	1	1	0	0	1	5	3	6
15	2	5	5	3	7	2	4	6	5	1	7	3	5	1	4	0	0	1	5	3	6
17	2	5	5	4	7	3	5	6	6	1	7	5	6	2	4	0	0	1	5	3	6
19	4	6	6	4	7	6	6	6	6	5	7	6	7	5	6	0	2	3	6	5	6
21	5	6	7	6	7	6	7	6	6	6	7	7	7	5	7	1	3	5	6	7	7
31	6	6	7	7	7	6	7	6	7	6	7	7	7	6	7	2	5	5	7	7	7

** The number of tomato plants, out of seven, wilted on a given day.*

contrast to this. In this group there was 100% infection in 31 days and wilting was as rapid as in plants transplanted into the recently infested soil. A slight lag in the development of symptoms is shown also by those tomatoes that were transplanted into soils that were infested on June 10 and cropped. This lag existed only for one week. Even though these soils were watered twice a day they did not contain as much moisture at the beginning of the final test as the soils that were left fallow and moist. Under cropped conditions it was not possible to cultivate the soils so that they became hard and cracked, particularly the two clay loam types.

From these three tests it is apparent that the fungus does not survive to the same extent in dry soils as in moist ones and its level of aggressiveness in the former as judged by the lag in time for the development of symptoms in tomato is lower than in soils containing abundant moisture. The presence of an immune or a susceptible host growing in infested soils over a three month period had no noticeable effect upon the survival of the fungus. Over this three month period no one of these soil types affected the survival or activity of the fungus more than any other. Since temperature of the soils in the different series was approximately the same over this period of time, it would appear that moisture is most important in controlling the activity of *Verticillium*.

Other Factors Influencing the Activity of Verticillium in the Soil

1. The Effect of Soil Moisture upon the Activity of Old Inoculum

Since soil moisture appeared to be influencing the activity of *Verticillium* to a great extent, an experiment was designed in which recently infested dry soil was watered for periods of varying length before the fungus aggressiveness was tested. In this experiment the inoculum consisted of a sand–cornmeal–Verticillium mixture which was five and one-half months old. The inoculum, previous to addition to the soil, was examined under the microscope. It consisted of masses of black "microsclerotia" with no hyaline, vegetative hyphae evident. Sufficient sterilized soil to fill flats *A*, *B*, and *C* was infested with 6% of its volume by mixing thoroughly this inoculum through it. The moisture content of the soil at this time was approximately 15% of its moisture holding capacity. The soil in Flat *A* was watered immediately and was given subsequently a copious supply each morning. The soil in Flats *B* and *C* was not watered until five and 10 days respectively after *A* was watered for the first time. Just previous to the initial watering of soil in Flat *C*, 13 small tomato plants were transplanted into each flat. Observations were made each day of the number of plants showing symptoms of wilt.

The results are shown in Table V. Plants in Flat *A* showed wilt symptoms first, followed a few days later by plants in Flat *B*, and again after a longer interval by those in Flat *C*. The numbers of plants showing wilt at the end

TABLE V

COMPARATIVE TESTS OF ACTIVITY OF *Verticillium* IN INFESTED STERILIZED SOILS WHICH HAD BEEN WATERED FOR DIFFERENT PERIODS OF TIME

Number of days after transplanting	Number of plants, out of 13, showing wilt in flats		
	A[1]	*B*[2]	*C*[3]
12	1	0	0
15	3	1	0
18	4	1	0
20	5	2	0
23	6	5	0
24	6	5	2
29	6	7	2
32	10	9	4
34	10	9	5

[1] *Flat A was watered 10 days before tomatoes were transplanted into it.*
[2] *Flat B was watered five days before tomatoes were transplanted into it.*
[3] *Flat C was watered at time of transplanting tomatoes into it.*

of 34 days were 10 in Flat *A*, nine in Flat *B*, and five in Flat *C*. At this time the plants were becoming so crowded that they were removed, the soil was cultivated and 15 tomato plants of uniform size were transplanted again into each flat. Table VI presents the wilting recorded in this test.

TABLE VI

COMPARATIVE TESTS OF THE ACTIVITY OF *Verticillium* IN SOILS IN FLATS *A*, *B*, AND *C* 34 DAYS AFTER THE FIRST TEST WAS BEGUN

Number of days after transplanting	Number of plants, out of 15, showing wilt in flats:		
	A	*B*	*C*
10	2	0	2
11	4	2	4
12	5	4	4
14	6	4	5
17	8	7	6
18	8	8	6
20	10	9	8
22	10	10	9

The second test reveals that there was no appreciable difference in the number of plants wilting in the three flats—*A*, *B*, and *C*. From the incidence of disease in the first test it would appear that inoculum in a resting condition when incorporated into the soil is not capable of parasitizing plants until it is activated. This can be brought about by the addition of moisture to a soil medium and would seem to be associated in part at least with the germination of resting elements of the mycelium.

2. The Influence of Adding Various Organic Substances to the Soil upon the the Aggressiveness of Verticillium albo-atrum

It is known that the microbiological balance of a soil is influenced by the available organic content. The predominance of certain groups of micro-organisms at a given time depends in part upon the readiness with which they utilize the available substrate. By changing the substrate the microbiological balance of the soil is altered in favour of those organisms that can best use the new substrate and the activity of other groups may be suppressed thereby. The substrate may be changed by adding organic materials to the soil. In this study organic materials consisted of: (*a*) green plant residues; (*b*) organic acids.

(*a*) To study the effect of plant residues on the activity of *V. albo-atrum* in soil the following experiment was conducted. To four flats of unsterilized

soil one of each of the following were added: 120 gm. (150 cc.) of rye, 120 gm. (150 cc.) of buckwheat, 120 gm. (300 cc.) of tomato, and 120 gm. (300 cc.) of snapdragon. The residues consisted of finely ground stems and leaves of young plants and were mixed thoroughly into each flat of soil. The soil was kept moist and 14 days later 100 gm. of sand–cornmeal–Verticillium inoculum was added to each flat of soil. A check flat minus the residues was infested at the same rate. Seven days later 21 small tomato plants were transplanted into each flat. Records of the number of plants wilting each day were kept for 30 days. At that time the survivors were removed, a second crop of young tomatoes was transplanted into the flats, and a similar record kept.

The results of these tests are presented in Table VII. There was little difference in the disease trends in the two tests. There was a slight lag in disease development in those plants growing in soils to which residues were added, with considerable differences in time of wilting in the various flats. There was no difference in size or type of growth of plants in the check series as compared with the plants in the other flats and it is assumed that this lag in disease development was due to a slight suppression of fungus activity. Since it was less pronounced at the end of the second experiment, this depressing effect does not seem likely to persist very long after the addition of a given residue.

(*b*) To study the effect of organic acids upon the activity of *V. albo-atrum* in the soil the following experiment was conducted. To one flat of non-sterile fine sandy loam soil 20 gm. of tartaric acid dissolved in a litre of water was added and to a second one an equal weight of citric acid. These two flats and another flat of the same kind of soil were infested with the usual *Verticillium* inoculum. A week later 21 small tomato plants were transplanted into each flat of soil. Owing to the high summer temperatures, atypical wilt symptoms were expressed so that the experiment was repeated in the fall. An additional 30 gm. of tartaric and citric acids were added to those that had previously been given these treatments. A week later tomatoes were transplanted into the flats. During the late fall season the amount of sunlight was not sufficient to promote rapid growth so that the experiment continued for 55 days and the number of plants wilting on the bright days was noted.

The results of the experiment are shown in Table VIII. With the exception of the first two observations more plants were wilting in the check flat each day than in either of the other two flats. At the end of the experiment 91% had wilted in the check flat whereas 77 and 58% respectively had wilted in the tartaric and citric acid treated soils. In a later test it was found that the addition of either citric acid or tartaric acid to soil in the same proportions favoured the growth of *Verticillium*. To explain the slight lowering of fungus activity in these soils it would appear that these organic acids favoured the growth of other components of the soil flora which in competition must have slightly suppressed the *Verticillium*.

TABLE VII

EFFECT OF VARIOUS PLANT RESIDUES IN THE SOIL ON FUNGUS ACTIVITY

Number of days after transplanting	Number of plants, out of 21, wilting in soils to which the following residues were added:				
	Rye	Buckwheat	Snapdragon	Tomato	Check
First planting					
7	0	0	0	0	2
8	1	0	0	0	3
10	2	3	0	0	4
13	4	5	2	1	8
14	4	6	7	4	11
15	7	8	9	7	13
17	9	8	10	7	16
23	13	12	14	9	20
30	15	12	14	11	20
Second planting					
8	0	0	0	0	1
9	0	0	0	1	3
11	0	0	2	1	5
12	1	0	2	2	8
13	1	1	3	2	10
14	3	5	3	3	10
15	5	5	5	9	13
17	7	5	9	11	14
20	8	7	12	13	14
23	9	7	13	15	15
24	12	12	14	15	17
30	13	13	17	18	18

The Phenology of Epidemic and Non-Epidemic Years

The charts presented in Figs. 4 to 9 summarize meteorological data obtained at the Horticultural Experiment Station, Vineland, Ont., with respect to May, June, July, and August of the non-epidemic years 1939 and 1941 and of the epidemic year 1940. The minimum and maximum air temperatures

TABLE VIII

EFFECT OF ORGANIC ACIDS IN THE SOIL ON THE ACTIVITY OF *Verticillium*

Number of days after transplanting	Number of plants, out of 21, wilting in soils		
	Check	Tartaric acid added	Citric acid added
17	1	2	0
31	5	5	0
34	7	5	0
40	13	6	2
42	13	7	2
43	13	9	4
45	16	11	5
47	17	11	5
49	18	11	7
52	19	12	8
55	19	16	12

and precipitation are included in all the charts and, in addition, the minimum and maximum soil temperatures at the 4 in. level are shown for much of the 1941 period. Where a comparison was possible, the minimum and maximum soil temperatures at the 4 in. level did not manifest such extremes as characterized the air temperatures, but tended to fall between the latter. Also, the average soil temperature at this level was slightly higher than the average air temperature for a corresponding period. This agrees with the report by Kimball, Ruhnke, and Glover (3) that in a light sandy soil the average monthly air temperature was slightly lower than the soil temperature at a 4 in. level.

From the data for the epidemic year 1940, it is evident that a distinctly high soil moisture must have been maintained throughout the entire growing season. The soil temperature was low throughout May and most of June and until July did not reach for any extended period the range which from the cultural studies one would expect to be conducive to fungous activity. It was at this time that wilt symptoms began to appear. If the non-epidemic years 1939 and 1941 are compared with 1940, it is seen that there were only slight differences in soil temperature. In 1939 and 1941, the temperature range during May was slightly broader than in 1940, but still remained rather low, from 45° to 60° F., whereas toward the end of June there was a slow rise to the 65° to 70° F. range. It was only in July and August that the temperature range was well suited to *Verticillium*. With respect to soil moisture, however, the years varied significantly. Subnormal precipitation was a conspicuous feature of both non-epidemic years in May and June; in July

and August of 1941 the precipitation continued subnormal whereas in 1939 there was more precipitation in those months than in the corresponding period of the epidemic year 1940. It is suggested therefore that both soil moisture and temperature operate in limiting the activity of *Verticillium* in Niagara Peninsula soils, low temperatures confining it to the latter half of the growing season and high moisture maintained throughout the whole season being prerequisite to any marked activity.

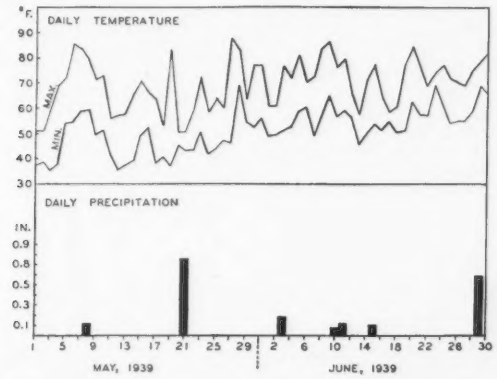


FIG. 4. *Summary of air temperatures and precipitation for May and June of 1939.*

Further evidence in support of this thesis was obtained in 1942 when *Verticillium* was again very active. Wilt symptoms were evident in several hosts, including peach, raspberry, sweet and sour cherries, roses, barberry, ash, and snapdragons, but once again they did not appear until late June or early July. In one two-year-old peach orchard 60% of the trees were infected, 30% of which were dead by the first of August. An analysis of the

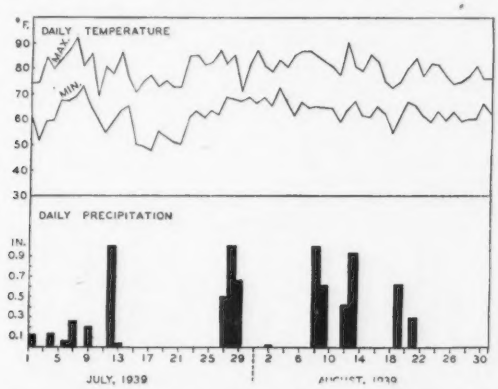


FIG. 5. *Summary of air temperatures and precipitation for July and August of 1939.*

phenological data of 1942 indicated a striking similarity to that of 1940, the other epidemic year. The soil moisture was extraordinarily high in May and rather high in June, with the precipitation uniformly distributed over each month. In July the precipitation was average, in August it was subnormal, nevertheless, the surface soils contained a considerable amount of moisture for most of this month. The soil temperature was low in May but rose to a

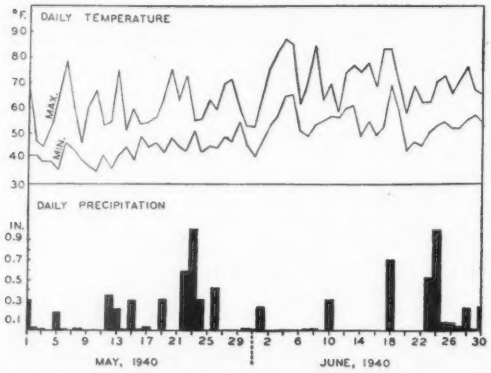


FIG. 6. *Summary of air temperatures and precipitation for May and June of 1940.*

level favourable for *Verticillium* activity late in June. Toward the end of June the first symptoms of wilt were recorded and throughout July and August appreciable amounts of wilt were apparent in many hosts.

Discussion

The pronounced effect that soil moisture had upon the activity of *Verticillium* in many of the experiments reported in this study supports the con-

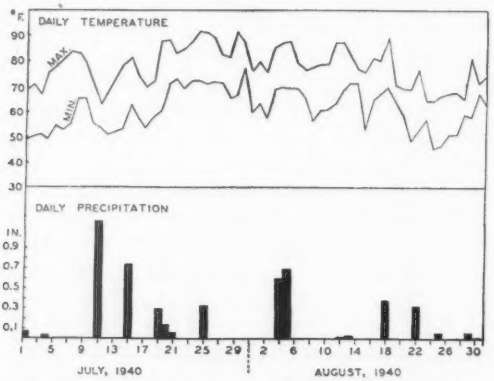


FIG. 7. *Summary of air temperatures and precipitation for July and August of 1940.*

clusion arrived at from an analysis of moisture conditions in epidemic and non-epidemic years that it is extremely important in favouring or inhibiting the development of disease. The temperature factor, however, cannot be ignored. The study of disease incidence in the temperature tank series with tomato as a host revealed that the maximum wilt appears at 24° C., and that disease begins to be serious only above 18° C. From the charts it is evident

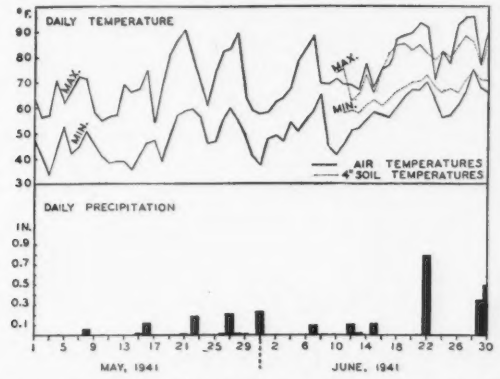


FIG. 8. *Summary of air and soil temperatures and precipitation for May and June of 1941.*

that temperatures suitable for fungus activity are not encountered ordinarily until late June and early July. At optimum soil moisture the amount of disease decreased more rapidly from a maximum at 24° C. than at high soil moisture. The suggestion appears to be, therefore, that an epidemic outbreak of *Verticillium* is contingent upon a high level of soil moisture maintained fairly uniformly throughout the growing season, even before soil

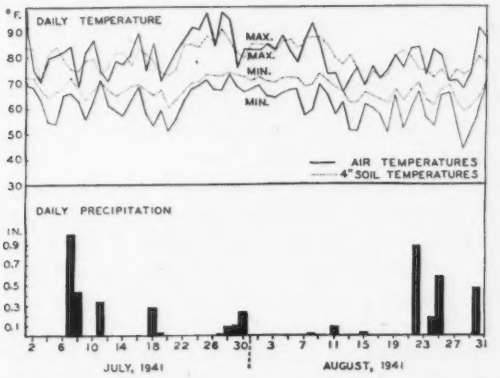


FIG. 9. *Summary of air and soil temperatures and precipitation for July and August of 1941.*

temperatures are high enough to permit disease development. This is suggested not only by the phenological data of 1940 and 1942, but also by the absence of an epidemic in 1939, when the soil moisture rose in July and August well above the average from a definitely subnormal level in May and June. The postulated requisites would go far to explain why serious outbreaks of disease occur so rarely on the Niagara Peninsula. From the data at hand it is not apparent whether infection occurs abundantly in the early part of the season and remains abortive until higher temperatures pertain, or whether high soil moisture in the early part of the season merely activates the fungus in the soil, with infection being initiated only later when the soil becomes warm. Further investigation should settle this point.

Since the temperature optima for disease incidence in tomato and for vegetative growth of the fungus in culture coincide, it would seem that the temperature factor influences the degree of disease incidence primarily through its effect upon the fungus. This is suggested further by the fact that symptoms appear in the field at about the same time in the various hosts, some of which are favoured by high and some by low temperatures, which indicates that the plants are not predisposed to attack by certain temperature conditions.

Although the disease epidemics in 1940 and 1942 occurred in seasons when the precipitation was much above normal and in experiments moisture and temperature are shown to play an important role in disease development, it seems scarcely possible that these two factors are operating simply and directly on the fungus itself. The composition of the microflora of a given soil merits consideration. The results of the preliminary experiments on adding organic substances to the soil indicate a slight temporary suppression in the activity of *V. albo-atrum* and this observation is in agreement with that of various investigations on other diseases. It is known that the addition of green manure to the soil brings about a striking increase in the bacterial population. Mitchell, Hooton, and Clark (4) found that sclerotia of *Phymatotrichum omnivorum* as well as mycelium are susceptible to elimination from soil during periods of intensive microbial activity following applications of fresh organic residues. The growth and survival of mycelium and "microsclerotia" of *V. albo-atrum* is determined to some extent by the competitive effects of other micro-organisms which are also in part a function of moisture and temperature. Competition may thus be a factor in influencing the aggressiveness of the fungus in any given soil.

The question of soil types that are favourable to fungus propagation needs further elucidation. Over a three month period it was found that *V. albo-atrum* persisted equally well in heavy red clay loam, medium clay loam, and in light sandy loam soils, but it would be interesting to study the activity of the pathogen in these soils over a more extended period. It has been observed in the Niagara Peninsula that the disease has been particularly common on light sandy loam soils. Many previous investigators regard loams and sandy loams as favourable for Verticillium disease, but it has also been reported as

common in gumbo soils in the Mississippi River. Garrett (2) says: "It appears that the majority of soil-borne fungous diseases are favoured by soils of light texture. Counts of *Fusarium cubense* in soil adjacent to diseased plants gave good correlation both with the severity of disease and with soil type, numbers increasing in a regular manner with increasing percentage of sand and decreasing percentage of clay in the soil." A similar study of the various soils throughout the Niagara Peninsula might reveal some important facts of the nature of distribution of *Verticillium*.

Finally, there is a suggestion that, in some if not all of the hosts, a further factor in disease incidence is a varying susceptibility correlated in an as yet unknown way with the developmental rhythm of the individual. Thus, only rarely do seedlings reach the completely wilted stage and in snapdragons wilt appeared only as the plants approached the blooming period. Furthermore, Verticillium wilt seems of no consequence in nursery stocks of peach and cherry and in one case under observation this season peach seedlings were not attacked under conditions where adjacent three-year–old trees were 100% infected and much older trees were also free from attack. It seems questionable whether under conditions in the Niagara Peninsula either potatoes or tomatoes ever even approximate the completely wilted stage and yet *Verticillium* can be isolated frequently from either potato or tomato plants that have chlorotic lower leaves and are lacking in vigour. It is thus apparent that the epidemiology of Verticillium wilt is not a simple function of appropriate soil moisture and soil temperature for fungous activity, but is complex and requires much further analysis for its elucidation.

Acknowledgments

The writer wishes to express his gratitude to Professor D. L. Bailey for his constant assistance and encouragement during this investigation. Thanks are also due to Professor E. F. Palmer for his co-operation in placing at the writer's disposal the facilities of the Horticultural Experiment Station, Vineland, Ont.

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Canadian Journal of Research

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

VOL. 21, SEC. D. MARCH, 1943 NUMBER 3

THE STANDARDIZING OF A LABORATORY METHOD FOR COMPARING THE TOXICITY OF CONTACT INSECTICIDES[1]

BY FRANK O. MORRISON[2]

Abstract

Toxicity tests were conducted with nicotine sulphate and nicotine alkaloid using *Drosophila melanogaster* as the test animal, with a modified Tattersfield atomizer spray machine, and by an immersion technique. One hundred and fifty flies were treated at each concentration each day. Each experiment was replicated 8 or 10 times using 3 to 22 concentrations. Data were analysed by the method of analysis of variance and by means of probits.

It appears from the data secured that careful standardization of any technique will be needed to secure comparable results. Results from spraying were the more uniform and consistent. Saponin spreader had a synergistic action with nicotine sulphate. It complicates results and its effect cannot be separated from that of the insecticide. Variations in observed mortalities result from different rates of spray application (slower applications were better), different ages of test animals (day-old flies and flies over four days old were most susceptible), different numbers of test animals per container (increased numbers increased the kill), different populations (these vary greatly in susceptibility), differences in larval and adult nutrition, and the use of different sized fly containers. All these factors must be standardized or accounted for. When this was done variations due to different experimenters were not significant.

In general six or eight replications were enough to establish a curve. Analysis of variance on angular transformation values gives a good test for consistency and the method of probits reveals much heterogeneity in the data.

Introduction

Tattersfield (36) has briefly but effectively reviewed the history of biological methods of testing insecticides. There is no need to repeat it here. Since that review O'Kane (27) has published the design of a new toximeter which has as its important characteristic the ability to spray the liquid on the insect from two directions, but greatly limits the number of test animals that may be used, and there has appeared a very excellent paper by Potter (32) emphasizing, as the following work does, the need of detailed standardization. The greater uniformity of results secured by Potter is at least in part due to the fact that all the data for one test were secured on one population of test animal. Both spraying and dipping methods have been used at Macdonald College for a number of years and a large accumulation of data has resulted. It is proposed to present much of these accumulated data in brief graphical and tabular form and to discuss them in the light of other published work.

[1] *Manuscript received in original form July 8, 1942, and as revised, December 4, 1942. Contribution from the Faculty of Agriculture, McGill University, Macdonald College, Que. Macdonald College Journal Series No. 175.*

[2] *Lecturer.*

Technique Used

Rearing and Handling of the Test Animal, Drosophila melanogaster

Bananas, partly peeled, dusted with dried brewer's yeast and placed horizontally on top of sawdust in candy-jars, and potato–yeast medium similarly used (34) proved the most successful rearing media. Other media (25) were not satisfactory for rearing large numbers of flies. Moreover, with the banana medium it was possible to add new food every few days and keep the nutritional condition of test animals reasonably uniform. Preliminary tests showed flies from exhausted media to be very susceptible to treatment. This effect of nutrition on susceptibility has been previously noted (24 and 31).

It was found convenient to cut out a portion of the tin tops of the candy-jars (Fig. 13) and replace it with fine copper wire gauze. Cultures were kept in chambers at 70° F. and approximately 80% humidity. New cultures were started by the addition of at least 500 flies to a newly prepared culture bottle. The population was allowed to build up (bananas being added as needed) for two weeks, but flies were not utilized for tests more than one month after the culture was started.

A candy-jar, the bottom of which had been replaced by copper wire gauze, was used as a collecting jar when it was desired to secure flies for tests. The top of a culture jar was unscrewed and a cover of two flat pieces of glass placed on it. Over these the collecting jar was inverted, the glass plates were withdrawn, and the culture jar and the inverted collecting jar were placed under a light, which induced the flies to enter the top jar. By manipulation of the two glass plates the collecting jar and the flies it contained could be removed and the culture left covered.

The flies were then blown, rather than shaken, into a glass separatory funnel through a cone-like adapter which fitted over the mouth of the collecting jar. When more than one culture was utilized for one experiment, all the flies were first blown into a reservoir jar and withdrawn as needed. This assured uniform sampling.

The separatory funnel could be held at a slight angle beneath an electric lamp and the flies crawled, when the large bore stopcock was open, into the outlet tube. This tube had been cut off about one inch beyond the stopcock and over it was placed a rubber collar which fitted snugly into the end of the small tubular fly containers. Flies might be easily counted as they passed the stopcock which acted as a gate to regulate the numbers passing.

The tubular fly containers were cut from glass tubing and were 45 mm. long by 14 mm. in diameter, inside measurement (Fig. 13). The flies were kept in the tubing by pieces of tulle, 28 mesh to the inch, stretched over the ends and held on by rubber bands. It was found advisable to preshrink new tulle for 24 hr. in 10% potassium hydroxide solution. One end of each container was covered prior to filling and the second end covered on removal from the collar of the separatory funnel.

When 150 flies were sprayed at one time in one container the container used was 22 mm. in diameter and 2½ in. long and the homeopathic vial into which treated flies were transferred was correspondingly large. Otherwise the handling was identical. When large numbers of flies had been immersed in one container they were transferred to filter-paper–lined Petri dishes rather than to homeopathic vials.

In all experiments except those specifically designed to test flies of different ages, flies used were from a few hours to four days old, the cultures being cleared of flies every four days. When flies of known age were required cultures were cleared each day and the flies retained in extra jars with a plentiful available food supply until required.

After being sprayed, specimens remained in the fly containers (which were moist with spray on all sides and through on the lower cover) for 30 min. during which time they were stored at 70° F. The flies were then removed by shaking or with a camel hair brush and placed in a homeopathic vial of the same diameter as the container. The vial was plugged with absorbent cotton previously dipped in a 5% honey solution and partly wrung out. Plugs were dipped just before use because, if dipped the day before, fermentation set in and the flies were stupified by gas accumulation in the vials. Mortality counts were made after 24 hr. storage at 70° F. All flies capable of movement were recorded as alive.

Spraying Technique

Spraying was carried out with a Tattersfield improved type atomizer (35) operated by a compressed air line (Fig. 13). A one-quarter h.p. motor supplied power to operate a small air pump, the air stream being directed through a reducing valve and filter to the atomizer and the pressure being measured by an attached manometer. An adjustable stand, glass fume cabinet, and clamp in which the containers could be supported directly beneath the cone of spray at a distance of 27 to 30 cm., as shown in Fig. 13, completed the apparatus. Spraying was carried out at a pressure of 15 lb. per square inch and the atomizer regulated to deliver 1 cc. of liquid in 20 sec.

Immersion Technique

Shell vials of the same diameter as the tubular fly cages used in spraying tests were utilized. Flies were counted into these and imprisoned by a loose fitting plug of non-absorbent cotton pushed down to near the bottom. The material to be tested was introduced by means of a drawn out glass tube with a syringe bulb at one end. It was withdrawn the same way. The fluid was allowed to rise above the plug, thus all specimens were submerged. All specimens were kept immersed for 30 min., a period that Gilbert and Marshall (19) found equivalent to spraying with 1 cc. and subsequent storage for 24 hr.

Morley (26) carried out immersions by submerging the flies in the regular spray-type of fly container, which was immersed in a large beaker of solution. Submersion was maintained for 20 min. instead of 30.

Making up Dilutions of Nicotine

The nicotine used was in the form of nicotine sulphate, as put out by Canadian Industries Limited, in commercial pound tins and labelled 40%, and in the form of the alkaloid put up by Kentucky Byproducts Co. Ltd., under the trade name of Nicofume and labelled 40%. On opening a new tin the concentration of nicotine was tested by the standard A.O.A.C. method of analysis (precipitation with silicotungstic acid, etc.). This analysis was repeated at intervals over a period of time. Attempts of the earlier workers to make up 20% stock solutions were given up as precipitates formed which clogged the atomizer. Each day a 2 or 4% stock solution was prepared from the tin, using the determination figures secured in the analysis, and the desired concentrations made up from this. Concentration throughout is expressed uniformly in weight (gm.) of the alkaloid nicotine present per 100 cc. of solution.

Design of the Experiments

In general the scheme adopted was to test the flies in lots of 15. Ten fly containers, each of 15 flies, were tested at each concentration. Where possible sufficient concentrations were used to give a picture of the complete range of toxicity. All concentrations were tested on the same day (same population) and the experiment replicated on 10 different days. Ten containers were treated with distilled water as a check. Modifications of this plan were necessary on one or two occasions. These are indicated in the following section. The object was to secure results on statistically significant numbers.

The following are the materials tested, and other phases of the subject investigated.

1. *By the Spray Technique*
   - *a*. Nicotine sulphate (11).
   - *b*. Nicotine sulphate in a 1% saponin solution.
   - *c*. Nicotine alkaloid ("Nicofume").
   - *d*. Nicotine alkaloid ("Nicofume") in a 1% saponin solution.
   - *e*. Nicotine sulphate in 1% saponin solution on flies of different known ages.
   - *f*. Nicotine sulphate sprayed at different rates.
   - *g*. Nicotine sulphate sprayed on 10 containers of 15 flies each and on one container of 150 flies.
   - *h*. Nicotine sulphate, saponin, and nicotine sulphate plus saponin tested on samples of the same population to determine if the saponin in combination acts synergistically.
   - *i*. Nicotine sulphate on fly samples reared on bananas and on potato–yeast medium.
   - *j*. Nicotine sulphate used on containers of 5, 10, 15, 30, 75, and 150 flies, respectively.
   - *k*. Nicotine sulphate tested by five experimenters on the same fly populations.
   - *l*. Nicotine sulphate tested on fly samples sprayed in containers of different diameters.

2. *By the Immersion Technique*

a. Nicotine sulphate.
b. Nicotine sulphate using 10 containers of 15 flies each and one container of 150 flies.
c. Nicotine sulphate in a 1% saponin solution.
d. Nicotine alkaloid ("Nicofume").
e. Nicotine alkaloid ("Nicofume") in a 1% saponin solution.

Discussion

The Evaluation of Mortality Data

It was the purpose of this investigation to study the standardization of a technique for comparing contact insecticides. It was recognized that the test animal had no economic importance, and that the results could have no direct application to control work.

In order to ascertain the value of the technique some method of the appraisal of results is essential. Tattersfield (36) has summarized the literature on methods of assessing results. He suggests that toxic action may be judged in three ways: "(*a*) by the effect produced by different concentrations in a given time, (*b*) by the effect produced at different intervals of time, the concentrations being kept constant, (*c*) by the effects produced at different intervals of time by different concentrations." The first of these, (*a*), giving rise to dosage–mortality curves is our only concern here.

To test our technique we should have some method of testing the consistency of results from different replications and from repetitions of complete experiments.

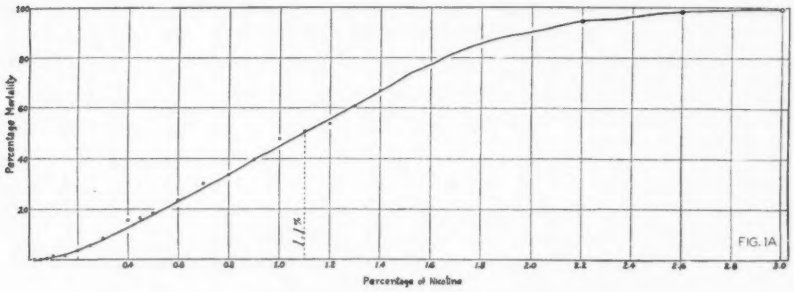
Cameron and Prebble (9) considered the per cent mortality secured from each container of 15 flies as a normal variate and worked out from these a standard deviation of the mean value of each mortality for each experiment. These formed a test of consistency, but were subject to errors arising from slightly different numbers in different containers from which the percentage mortalities (later averaged) were derived. It was suggested by these workers and by Dr. Hopkins of the National Research Council that if each concentration were tested each day, the percentage mortality for each replication (percentage derived from the totals dead and alive at each concentration, i.e., in 10 containers) might be treated as a normal variate, and the entire experiment subjected to analysis of variance. This set-up was adopted by the writer. However, on completing the work, and on further correspondence with Dr. Hopkins, it was concluded that the analysis of variance procedure should not be applied to data in which the percentage kill varies from 0 to 100, since the expected variance at the different dosages would not be the same (1, 6, 7, and 12). In order that the whole of the observations may be employed in the estimation of a common error, which will thus be computed with considerable accuracy, each variate can be transformed to a value in terms of angles of equal information (6, 7) and as this equalizes the expected variance

from each treatment, the analysis of variance procedure is justifiable. It was pointed out by Dr. Hopkins that the design of the experiment whereby each dosage was tested each day allowed for the elimination of the sum of squares between replicates from the experimental error and greatly reduced this value.

The average kill for each concentration in terms of angles and the standard error of the mean kill for any concentration is shown for each experiment in the tables given. The value of this method as a measure of the consistency of the data is evident. Where different numbers of replicates have been tested, it measures the evident greater consistency with increased numbers. Applied to immersion and spray results, it will be pointed out that the data from the spray technique show the greater consistency.

Reproducibility of Results

A logical question asked by people who must make routine tests of comparative toxicity is, "How many times must each test be replicated in order to secure reliable results?" All attempts to answer this question have proved futile because of the marked inconsistencies between the results of successive tests. In one test, cited below, an increase of two replications actually increased the error of the mean. Under these conditions our search must be



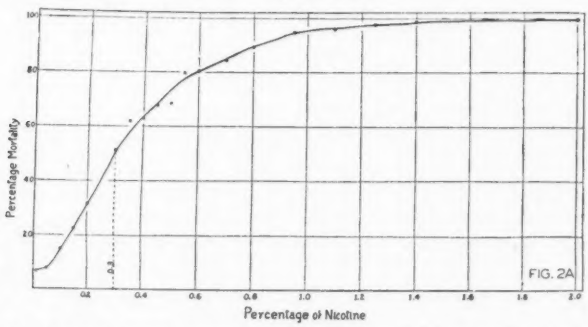


FIG. 1A. *Sigmoid–dosage–mortality curve of nicotine sulphate used as a spray (11).*

FIG. 2A. *Sigmoid–dosage–mortality curve of nicotine sulphate in a 1% saponin solution used as a spray.*

first for means of getting consistent results rather than for the optimum number of replicates.

Table I*d*, and Figs. 2A, 2B*a*, and 2B*b* are an attempt to throw light on this discussion. The first four replications, then the first six and the first eight have been compared with the 10 replications for one representative experiment. Any of the other experiments would, no doubt, show similar trends. If one merely examines the results and the sigmoid curve, it appears that increasing the replications gives progressively better results, i.e., by the time 10 replicates have been averaged each successively larger dosage gives rise to a higher mortality, and hence allows for a better fitting curve. This is as we would expect—the larger the number of populations sampled the more closely the averages fit a definite curve.

Each group of replicates was subjected to an analysis of variance and the resulting variance, expressed as angles of equal information, indicates the least variation of the data from the experiment mean when six replicates were used, the standard deviations of experiments of 4, 6, 8, and 10 replicates being 3.28°, 2.51°, 3.43°, and 4.59° respectively. When these are divided by the root of the number of variates in a mean to secure the standard error of the mean the order remains the same though 8 and 10 replications are then seen to have an advantage over four. The standard errors of the means work out to be 1.64°, 1.02°, 1.21°, and 1.45° respectively.

It is especially significant that in each analysis of variance the variation between replicates is significantly three or four times as large as the residual error. Similar figures for variance due to replications occurred in all the other experiments, as is shown by the following table.

Experiment	Variance between replicates	Residual error
Nicotine sulphate + saponin spray (4 repl.)	104.53	10.82
Nicotine sulphate + saponin spray (6 repl.)	277.05	6.29
Nicotine sulphate + saponin spray (8 repl.)	274.57	11.73
Nicotine sulphate + saponin spray (10 repl.)	335.48	21.09
Nicofume spray	137.15	15.72
Nicofume spray + saponin spray	489.16	17.70
Nicotine sulphate immersion	389.23	47.47
Nicotine sulphate + saponin immersion	267.63	19.08
Nicofume immersion	224.25	35.20
Nicofume + saponin immersion	90.97	24.78
Nicotine sulphate + saponin spray		
1 cc. per 10 sec.	267.35	27.18
1 cc. per 25 sec.	222.26	20.72
Nicotine sulphate spray		
15 flies per container	144.81	26.22
150 flies per container	141.78	49.59
Comparison of nicotine sulphate and nicotine sulphate + saponin	270.37	25.24
Comparison of different container diameters	134.27	43.46
Comparison of different workers	171.70	44.29

This indicates significant variation of the susceptibility of populations. It makes the completion of one replicate each day essential as the lower range of a curve calculated on today's population is not comparable with the higher range calculated tomorrow. Accurate comparative work involving the comparison of a material under investigation with one better known may have to be done on the same populations. Thus instead of a previously determined standard curve or one based on cumulative data, as suggested by Bliss, the standard material may have to be tested each time along with the new material on the same populations, as is done now in some biological assay work.

The variances in the above table are measured in degrees. Dr. Hopkins has pointed out that there is certain variation inherent in the value θ. This theoretical expected remainder, due to chance alone, is represented by the formula $V\theta = \frac{8100}{\pi^2 n}$. Assuming n in all tests conducted to be 150 (the number of flies tested per replication), $V\theta = 4.57$. If we examine the table, we will observe that the residual error variance varies from 6.29 to 49.59. In all tests except one it is over twice the anticipated value due to chance alone and in 13 out of 17 experiments it is well over three times this value. The technique can evidently be still further refined to eliminate the sources of this variance. What they may be we can only guess but slight variations in temperature during the experiment and unobservable differences in the methods of transferring flies from containers to vials, may account for some of it as may also varying sex ratios in different containers. Potter (32) presents evidence that indicates that at least some of this variation is due to "unequal administration of the dose" and he suggests that the microclimate around individual insects between spraying and counting may be an important factor.

Mortality–Dosage Relationship

It was early pointed out (40) that since the sigmoid–dosage–mortality curve tails off at its upper and lower ends, the best comparison of two insecticides is possible near the 50% kill point or so-called Median Lethal Dose or *L.D.* 50. This point is also discussed by Bliss (5). Clark (12) and others have discussed the interpretation of the sigmoid curve, and Bliss (3), O'Kane (28), Gaddum (18), Hemmingsen (21), and Bliss (4, 7, 8) have suggested conversion values that would reduce the curve to a straight line and make comparison based on the entire line (complete range of kill) possible. The tables of Bliss have been widely used and Cochran, in a supplement to Tattersfield's and Martin's paper (38), developed a numerical comparison of the toxicity of two insecticides.

In view of the wide acceptance of this method, a probit–log–dosage regression line has been fitted to each set of data secured by the writer. The methods including the weighting schemes given by Bliss (4) have been used. The variance of the position (Va) and the variance of the slope (Vb) are in-

dicated. In view of the much larger number of points determined and specimens used than in previously published data, the calculations involved proved an arduous task. If each mean for each replication were taken as a separate variate and thus the number of degrees of freedom for Chi^2 greatly increased the labour involved would also be increased, though such a method should be more accurate, as evidently populations vary considerably from day to day.

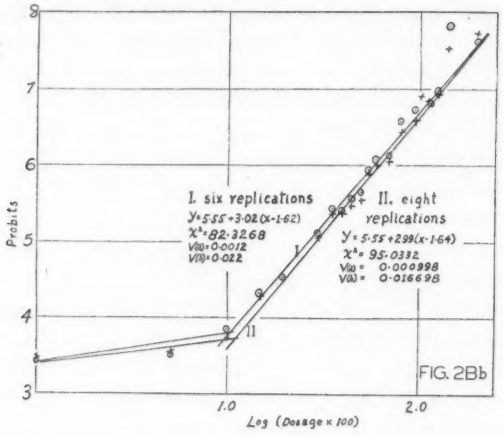
The consistency of the data from successive replications has been tested by this method in one experiment (Table I*c*). Using Chi^2 as the test of the goodness of fit, the results from the first four replicates conform more closely with the theory than do those of any larger number. When the first four and following four replications of the tests with nicotine sulphate and saponin used as a spray are considered as separate experiments and interpreted by the method of probits (5), the formulae 18, 19, and 20 (p. 316) of Bliss's article may be used to test the consistency of the two sets of data. When this is done (Figs. 2B*a*, 2B*b*, and 12), the Chi^2 for difference in position equals 208.376 and the Chi^2 for difference in slope equals 0.2203. It is thus seen that the lines have almost the same slope but differ widely in position and, in the words of Bliss, the new data "are not sufficiently consistent with the original experiments to warrant including them in a common dosage mortality curve." But these data are part of the same experiment during the progress of which there seems to have been a definite and steady decrease in susceptibility over the whole range and hence a shift of the regression line downward. It seems probable that the data from any of the other experiments would show similar inconsistencies.

When the regression line for each replicate in this set of data is calculated separately (Table I*c*), it will be observed that the slopes of the lines are reasonably parallel except for that of Replicate 10. Where the slopes of the lines of two materials differ widely, comparison of toxicities (or susceptibilities) may have to be made at some point other than the *L.D.* 50.

If we consider the data in Table IX and Fig. 10A where the same technique and material were employed but the rates of application were 1 cc. per 10 sec. and 1 per 25 sec. as compared to the rate used throughout other experiments (1 cc. per 20 sec.) the very differences in the *L.D.* 50's would indicate that these data are not comparable. Less easily justified inconsistencies or evidences of unexplainable variation are shown by the *L.D.* 50 of 1.1% secured by Cannon for nicotine sulphate used as a spray and the *L.D.* 50 of 0.89% secured by the writer with similar material and technique when the numbers of flies per cage were being tested, as well as by the inconceivable differences in the results with nicotine sulphate and saponin spray when tested first with different numbers of replications and later on flies of different ages.

In the face of this evidence it becomes difficult to say what constitutes the best number of replications. If these unexplainable variations are due to

seasonal effect on susceptibility or any other long term effect, the fact that would matter would not be the number of replications alone but the number related to the period of time required to conduct them. That the cause is a "long term" one is suggested by the much greater consistency of data from successive replications than of data from experiments conducted at widely different times. Potter (32) was unable to account for changes in the resistance of the population as a whole, in the course of a few generations.



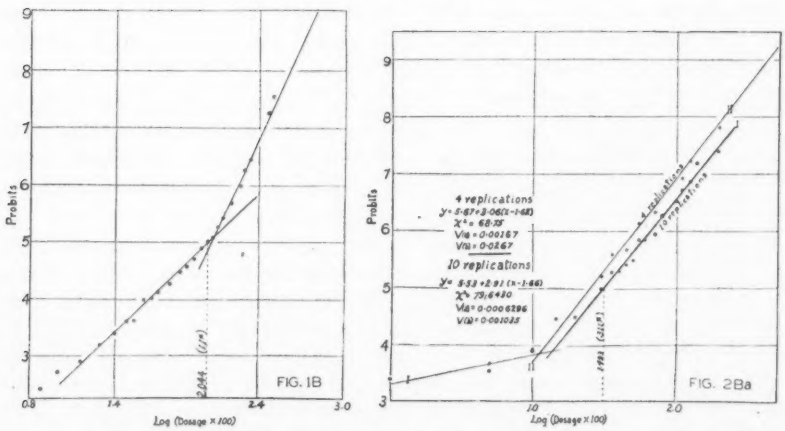
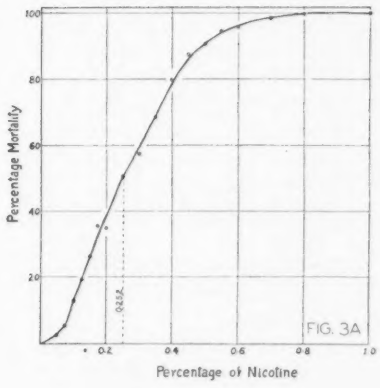


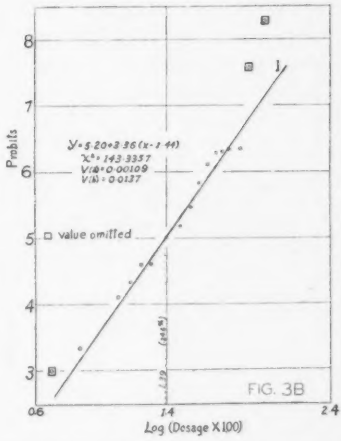
FIG. 1B. *Probit–log–dosage regression line of nicotine sulphate used as a spray fitted by eye to the data of Cannon (11).*

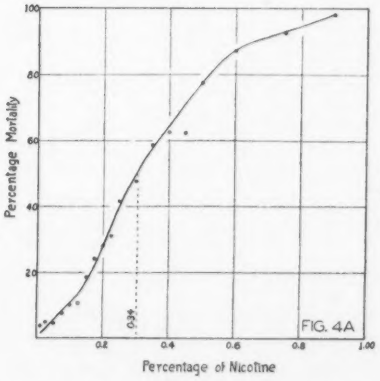
FIG. 2Ba. *Probit–log–dosage regression lines for nicotine sulphate in a 1% saponin solution used as a spray, when 4 and 10 replications are considered. (The lines were fitted by the method of Bliss (5) and the corresponding Chi² and variances are given.)*

FIG. 2Bb. *Probit–log–dosage regression lines for nicotine sulphate in a 1% saponin solution used as a spray, when six and eight replications are considered. In the expression, $y = 5.55 + 299\ (x - 1.64)$, read 2.99 for 299.*

Accepting Bliss's criterion for homogeneity, i.e., a Chi² not greater than the one for which the corresponding P in Fisher's Table III is 0.05, only the test involving nicotine sulphate and saponin sprayed on two-day-old flies shows this quality in the test animals. In that test only three points on the curve were determined. Heterogeneity of the data is thus generally indicated in all cases. In many experiments the very large Chi² suggests that the theory involved in the application of the method of probits probably does not describe the data and some curve other than a straight line would fit the converted data better. In fact the existence of variations from the regression lines is established. It is the nature of these variations that should be investi-







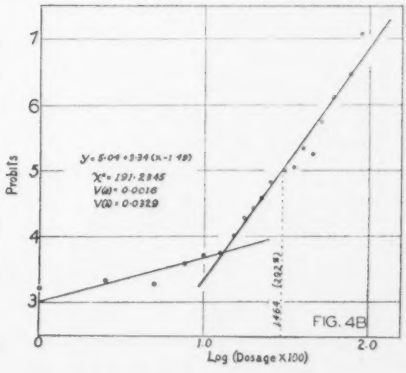


FIG. 3A. *Sigmoid–dosage–mortality curve for "nicofume" spray.*

FIG. 3B. *Probit–log–dosage regression line for "nicofume" spray.*

FIG. 4A. *Sigmoid–dosage–mortality curve for "nicofume" in 1% saponin used as a spray.*

FIG. 4B. *Probit–log–dosage regression line for "nicofume" in 1% saponin used as a spray. In the expression, $y = 5.04 + 3.34\ (x - 149)$, read 1.49 for 149.*

gated. Thus the underlying relationship may be linear and these variations random in their nature and simply due to faulty manipulations and heterogeneous experimental material along with pure sampling errors, or the mortality–log–dosage relationship may not be truly linear so that a better fitting line would be obtained by including quadratic or higher order terms. It is the hope of the writer that these or similar data may, in the future, be worked over with this matter in mind.

Factors Specifically Tested

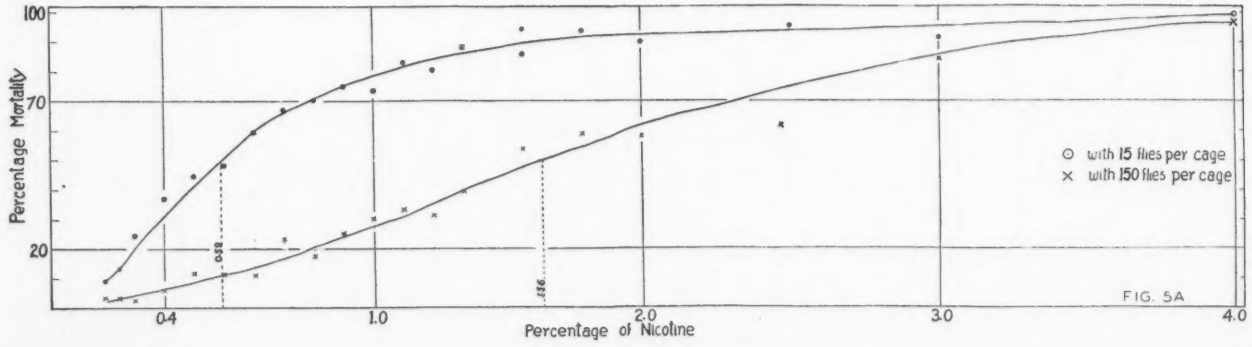
Several specific factors significantly affect the results secured in these tests. Nevertheless standardization of these factors alone does not seem to assure results reproducible within a desirably small experimental error. All experiments conducted exhibit a residual error sufficiently in excess of the expected random sampling fluctuations to suggest that in no test was a state of statistical control secured. In fact a statistical reviewer has pointed out to the writer that the title "standardizing of a laboratory method" is in truth a misnomer as standardization has not been effected. Further work is essential to refine experimental methods in such a way as to insure reproducible results within a predictable error. However, the extensive tests reported here throw much light on the difficulties involved in this type of test work.

The data secured lend themselves to the illustration of the following points:

THE COMPARATIVE VALUE OF SPRAYING AND IMMERSION TECHNIQUES

Experiment	(1) Standard deviation for the experiment by analysis of variance (in angles of equal information)	(2) Chi^2 derived from comparison of a fitted probit–log–dosage regression line with the data
	Immersion technique	
Using nicotine sulphate	6.90°	134
Using nicotine sulphate and saponin	4.39°	95
Using nicotine and saponin	5.02°	111
Using nicotine	5.88°	309
	Spray technique	
Using nicotine sulphate and saponin	4.59°	60
Using nicotine and saponin	4.21°	191
Using nicotine	3.95°	143

From the above table, Column 1, it is evident that data secured by the spraying technique were more consistent than those secured by immersion. Column 2, however, based on the probit scheme, fails to indicate any greater conformity of the data to the theory. The greater inconsistency in results from immersion probably arises from the difficulty experienced in getting the fluid uniformly in contact with the flies. A container of immersed flies, when held up to the light, always showed varying amounts of air held as



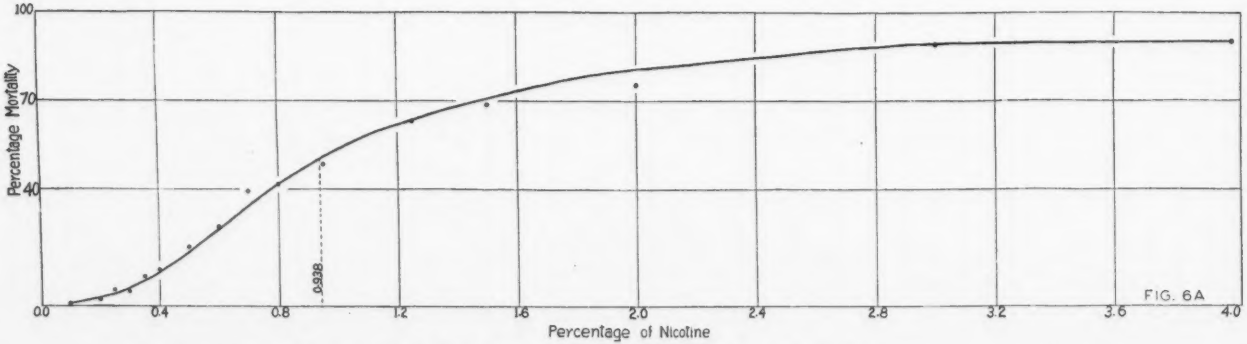


FIG. 5A. *Sigmoid–dosage–mortality curves for nicotine sulphate by the immersion technique.*
FIG. 6A. *Sigmoid–dosage–mortality curve obtained using nicotine sulphate in 1% saponin by the immersion technique.*

small bubbles by the setae of the flies. This might be overcome with different test animals (14). The retention of bubbles was especially evident when saponin was used. The spreader reduced the surface tension of the solution. It is especially noticeable that though it greatly enhanced the killing power of nicotine sulphate used as a spray, it did not do so to any extent when immersion was practised and with the latter technique 100% kill was very difficult to attain. It is possible that the reduced surface tension of the mixture did not allow sufficient of a film for the slow formation of gaseous nicotine from nicotine sulphate, to build up lethal concentrations before the film dried up.

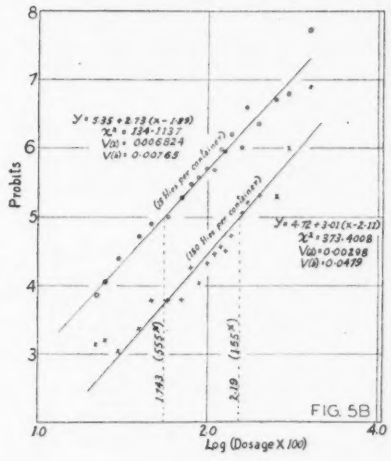
Table IV*b* has been included to illustrate how slight variations in technique between two workers may entirely vitiate results. If any one row of the results in this table is examined, a regular alternation of high and low values for successive concentrations may be observed, while the row above or below shows a similar alternation with concentrations that previously gave high kills now showing low ones. In order to complete all concentrations in one day, the writer and his assistant found it necessary for each to carry out treatments at the same time. To obviate any possible effect of this arrangement, it was decided that the concentrations tested by the writer one day would be tested by his assistant the next day. The techniques used came, in time, to differ slightly. After the immersion was complete, the fluid was removed with a glass tube and syringe bulb. The cotton plug was removed with forceps. Often many or all flies adhered to this plug. Excess fluid was drawn from the plug with the syringe. The flies were then transferred to the homeopathic vials. For this last process, the writer consistently used a comparatively dry brush with the excess water removed from it by pressing it on the edge of the beaker of distilled water (in which it was kept) each time it was removed. The mortalities thus secured were comparatively high. The assistant used a saturated brush the excess water from which was absorbed by the partially dry cotton plug. The mortalities secured were comparatively low. Presumably the water passing from the brush to the plug washed from the flies the adherent film of toxic substance. Correction of this variation resulted in uniform mortalities.

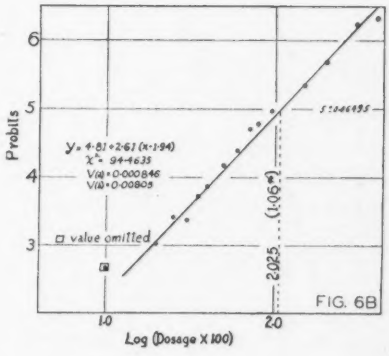
It is obvious that a test as sensitive as this would be difficult to standardize so that results might be duplicated by different workers.

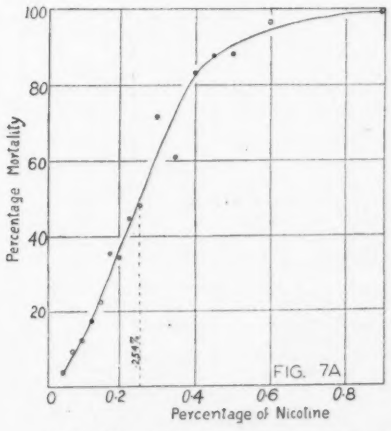
The Use of a Spreader

It is logical that introducing a third compound will complicate results. Saponin itself is toxic to *Drosophila*. In tests involving this spreader the toxicity of the saponin alone was tested. It gave an average mortality of 5%. Cameron and Prebble (9) corrected all their data on the basis of the "saponin check mortality" using the well known Abbott's formula. This correction assumes the spreader and nicotine to act separately. The present writer made no such corrections except for "water check mortalities", when these exceeded 3%. The Abbott correction, applied to those sets of data where the lower end of the probit graph fits poorly, would appear to improve this fit

(Figs. 5B and 7B). Saponin tested with nicotine sulphate by the spray technique increases the mortality approximately four times whereas used with "Nicofume" it raises the toxicity only slightly above that of "Nicofume" alone. On the other hand, nicotine sulphate alone proved more toxic than when used with saponin by the immersion technique.







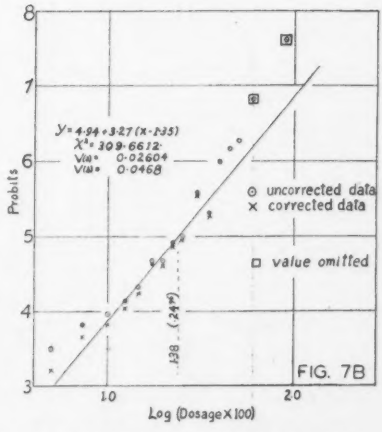


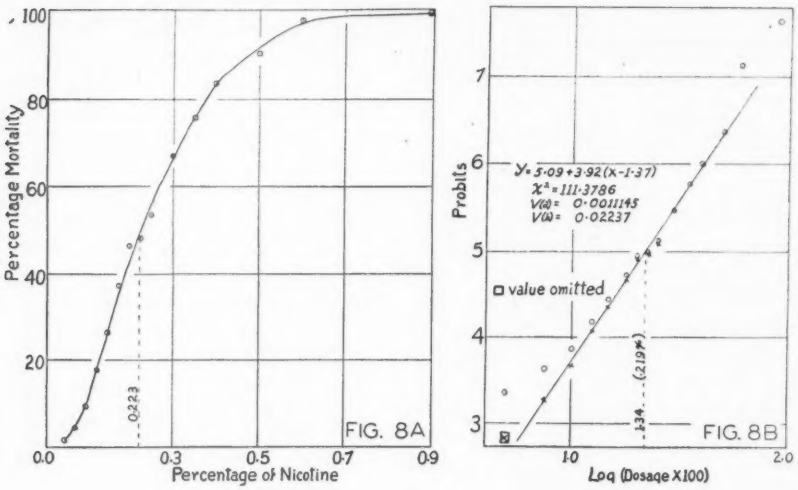
FIG. 5B. *Probit–log–dosage regression lines for nicotine sulphate by the immersion technique.*

FIG. 6B. *Probit–log–dosage regression line for nicotine sulphate in 1% saponin tested by the immersion technique.*

FIG. 7A. *Sigmoid–dosage–mortality curve obtained using "nicofume" by the immersion technique.*

FIG. 7B. *Probit–log–dosage regression line for "nicofume" tested by the immersion technique.*

Bliss (8) has discussed the phenomenon of synergism and the effect of different types of joint action on the form of the fitted probit–log–dosage regression line. If we compare Fig. 1B and Figs. 2B*a* and 2B*b* by Bliss's standards, we are inclined to see definite evidence of synergism in Figs. 2B*a* and 2B*b*. However, this sweeping statement is hard to justify since Fig. 1B



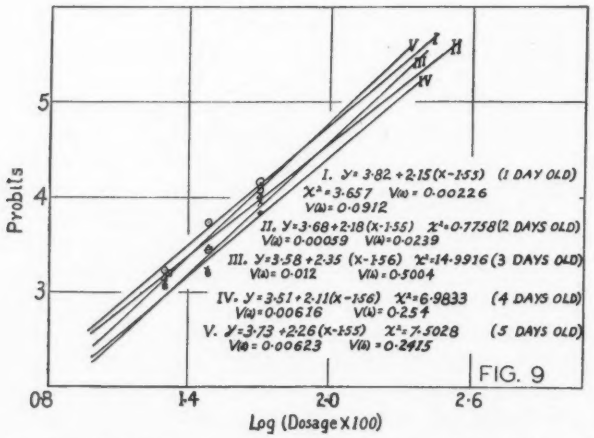


FIG. 8A. *Sigmoid–dosage–mortality curve obtained using "nicofume" in a 1% saponin solution by the immersion technique.*

FIG. 8B. *Probit–log–dosage regression line for "nicofume" in a 1% saponin solution by the immersion technique.*

FIG. 9. *Probit–log–dosage regression lines for nicotine sulphate in a 1% saponin solution tested as a spray on 1-, 2-, 3-, 4-, and 5-day-old flies.*

itself would appear to evidence the individual and separate action of two materials. Yet Fig. 10B compares favourably with Figs. 2B*a* and 2B*b* and gives evidence that results can be reproduced. The conclusion that there is a synergistic effect between the saponin and nicotine sulphate when used as a spray is borne out by the great increase in the toxicity when the mixture is used. This is in keeping with the work of Maxwell (24), who points out that various soap spreaders have a large adjuvant value with nicotine sulphate used as a spray but little when used with the alkaloid. When the immersion technique was used (Figs. 5B and 6B) the effect, though much less, must fall in either Bliss's Type 2 "similar joint action" or Type 3 "synergistic action".

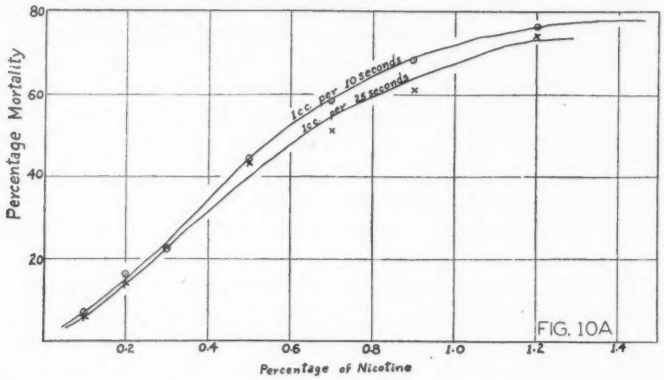
But now consider the "Nicofume" results. By the spray technique (Figs. 3B and 4B), it appears that the saponin may act independently, the upper part of the second curve being little changed, while by the immersion technique (Figs. 7B and 8B) no effect is evident.

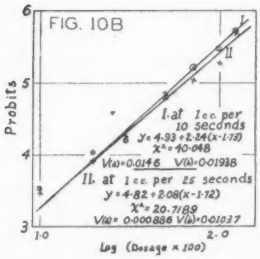
On the suggestion of Dr. Hopkins an experiment was set up to measure the synergistic effect of saponin used with nicotine sulphate. Eight replicates of 150 flies each (tested as before in containers of 15 flies each) were tested at concentrations of 0%, 1.1%, and 1.5% nicotine. Each concentration was used alone and in combination with 0%, 1%, and 3% saponin (Table XI). The resulting mortalities were transformed to Bliss's Angles of Equal Information, and set up as a 3 × 3 table, for analysis. The interaction between saponin and nicotine, if significant, should indicate a synergistic action. The following were the results:

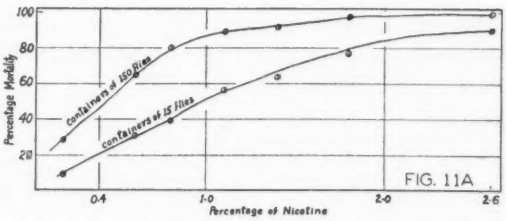
Source of variance	Sums of squares	Degrees of freedom	Variance	*F*	*F* for 5% *P*	*F* for 1% *P*
Variance for replicates	270.37	7	38.62	1.53	2.25	3.12
Variance for treatments						
(*a*) for conc. of nicotine	32561.47	2	16280.74	645.04	3.15	4.98
(*b*) for conc. of saponin	4218.41	2	2809.20	83.56	3.15	4.98
Variance for interaction (nicotine × saponin)	321.98	4	80.50	3.19	2.56	3.72
Error	1413.63	56	25.24			
Total	38785.86	71				

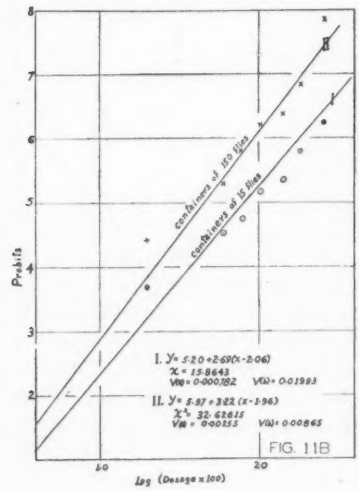
The interaction is seen to be within the range of significance. On examination of the data Dr. Hopkins has suggested that the interaction (hence degree of synergism) is much more pronounced than this analysis suggests. It is essentially the same for each concentration of the two tested. To show this a computation of the component of interaction variance ascribable to the single degree of freedom representing the difference in mortality between no nicotine and either 1.1 or 1.5% in the absence of saponin and in the presence of 1 or 3% saponin may be made.

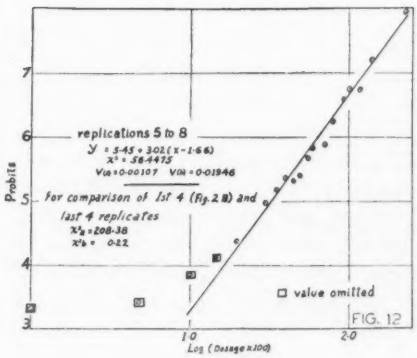
The interaction variance for this single degree of freedom accounts for no less than 277.14 of the total 321.98 and thus significantly exceeds the mean square error. It is thus established that in this experiment a measurably significant synergistic effect was apparent.











The addition of the saponin made regular and even spraying difficult as it interfered with the action of the atomizer. To explain the drop in mortality resulting from the same mixture as in Fig. 2B*a* (*L.D.* 50—0.311%), Fig. 9 (*L.D.* 50 from 0.12 to 0.22%), Fig. 10A (*L.D.* 50—0.46 to 0.63%), and Fig. 11A (*L.D.* 50—0.55 to 0.7%) preliminary tests were carried out on different samples of saponin varying from crude to chemically pure but no evidence of important variations in effect was obtained.

It is evident that we cannot gauge the nature or extent of the effect of a spreader on toxicity, and that it simplifies the comparison of insecticides when the spreader can be left out of consideration.

The Rate of Application

Figs. 10A and B indicate that the rate of application of the spray at least within the limits tested is not as important a factor, when animals as large and active as *Drosophila* are sprayed through tulle, as one might expect. The slower rate, however, gave the more consistent results from the point of view of the fit of a regression curve ($Chi^2 = 21$ for the slow rate as compared to 40 for the more rapid) and also gave data with less variance (S.d. 4.55° as compared to 5.21°).

The Age of the Test Animal

Cameron and Prebble (9) (Table VIII*c*) using flies not over three days old and flies not over four days old found considerable difference in susceptibility. These workers used nicotine sulphate plus saponin and secured kills comparable with those first obtained by the writer with these materials.

Maxwell (24) using flies of known ages found one-day–old flies much more susceptible than flies at other ages and three-day–old flies the most resistant forms tested.

Tables VIII*a* and VIII*b* and Fig. 9 indicate results secured in experiments by the writer. At a glance it is evident that the average kill given by what was previously the *L.D.* 50 was now less than 10%. No error could be found in the dilutions but it is expected one must have occurred. The nicotine sulphate when analysed gave the same percentage nicotine as before and used alone at 1.1% (*L.D.* 50 of Cannon's work) gave 58.6% kill. The laboratory temperature ranged 3° to 5° C. lower than during the earlier work. Any other variation would appear to be due to a new supply of saponin but varia-

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FIG. 10A. *Sigmoid–dosage–mortality curves obtained testing nicotine sulphate in 1% saponin solution by the spray technique.*

FIG. 10B. *Probit–log–dosage regression lines for nicotine sulphate tested in 1% saponin solution by the spray technique. For $V(a) = 0.0146$, read $V(a) = 0.00146$.*

FIG. 11A. *Sigmoid–dosage–mortality curves obtained using nicotine sulphate spray.*

FIG. 11B. *Probit–log–dosage regression lines for nicotine sulphate tested as a spray. For $\chi = 15.8643$, read $\chi^2 = 15.8643$.*

FIG. 12. *Probit–log–dosage regression line for nicotine sulphate in a 1% saponin solution used as a spray. Replications 5 to 8 inclusive are considered. The Chi^2 values for the comparison of the position and slope of this line with that for the first four replications (Figs. 2Ba and 2Bb are included). For Fig. 2B, read Fig. 2Ba.*

tions in the quality of the crude saponin used were not demonstrated by preliminary tests.

The greatest differences between any two ages occurred between one-day–old flies treated with 0.3% nicotine (kill 10%) and five-day–old flies treated with the same dosage (kill 5.9%). If each of the 10-percentage kills for the 10 replications be treated as a normal variate an error of the mean for each treatment may be secured. These errors being determined, we find the error of the difference between the two means to be 2.24%. The difference cannot be called significant. Similarly the mortality of one-day–old flies treated with 0.5% nicotine was 21.8%, while that of four-day–old flies was only 11.5% but the error of the difference between these two means is 8.10%. However, if the errors be determined simply as errors of sampling, the formula $\alpha = \sqrt{\frac{PQ}{N}}$ being used, the errors of these differences are much smaller.

The probit–log–dosage regression lines for these results have been calculated and drawn (Fig. 9). The goodness of fit of these lines varies and cannot of course be considered as valuable a test where only three points are used, as this measure of fit must obviously depend on one point. Moreover, with the low kills obtained, the lines may not be the true ones. It will be recalled (5) that the data secured at low concentrations are frequently inconsistent with the straight line hypothesis.

A second experiment was carried out at a later date using nicotine sulphate alone as a spray, and one concentration only, the *L.D.* 50 of Cannon's work (11), 1.1% (Table VIII*b*).

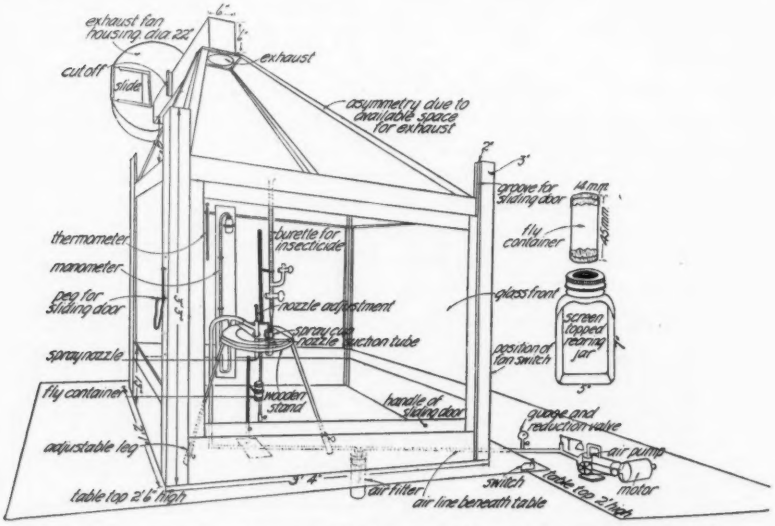

FIG. 13. *Glass cabinet housing modified Tattersfield atomizer, rearing jar, and fly container.*

It would appear that age affects susceptibility to some extent. Probably about 10% of the flies in each population used were older than the estimate owing to the impossibility of removing all the flies from a culture at one time. On two occasions results with certain populations showed unusually high susceptibility and investigation proved that the bananas supplied as food had hardened on the surface and were not available. These results were discarded, but the suggestion remains that nutrition is an important factor in governing susceptibility (24).

The Number of Test Animals per Container

It was the contention of Maxwell (24) that the treating of a larger number of flies in the same container would reduce the labour and the sampling error. The sampling error, however, is more dependent on the number of populations sampled than on whether the sample be treated all at once or in batches.

Tables IV*a* and IV*b*, Fig. 5A and Table X, Figs. 11A and B give results of experiments in which the treatment of 10 containers of 15 flies each has been compared with the treatment of 150 flies in one container. The same populations were sampled in each test. In so far as possible the techniques were similar for the containers of various sizes. Only 1 cc. of spray was used per container, regardless of the number of flies involved.

If we consider the results by immersion, the differences in the *L.D.* 50 and the dissimilarity in the curves suggest that variations in numbers per cage totally upset the results. If we accept the probit–log–dosage regression line interpretation the measure of goodness of fit shows the smaller numbers to be more satisfactory. When the spray technique was used the larger numbers resulted in a higher mortality as opposed to a lower mortality when immersion was practised. Again the small numbers gave the better fitting regression line.

A further investigation of the effect of the number of flies per container was attempted, using the standard containers normally loaded with 15 flies each. Tests were carried out at two concentrations of nicotine, 1.1 and 1.5% (used as nicotine sulphate by the spray technique) using 150 flies for each test, in lots of 5, 10, 15, and 30 flies per container. The larger containers used in the early tests when 150 flies were treated per container, were included with lots of 75 and 150 flies. The tests were replicated eight times (Table XII).

The steady rise in mortality as the numbers of test animals were increased would seem highly significant. Unfortunately any attempt to draw the probit lines for each test is bound to be disappointing as the slopes of such lines, determined from only two points are highly inaccurate. Thus the slopes determined from the above values vary widely. It would seem probable that determined from several points such lines might lie almost parallel. This, however, could not be tested as it was found impossible to carry out enough tests to determine five points or more on each of a number of curves

each day. It will be noted, however, that in Fig. 5B such parallelism is evident.

The rising mortality figures for any one treatment seem to bear an almost logarithmic relation to the increase in test animals per container. If this rise be graphed by plotting the numbers of flies on the abscissa against the mortalities on the ordinate the curve secured resembles the curves of absorption phenomena. Substituting the logarithms of the numbers of flies in the above graph does not give a completely straightened curve.

Effect of the Medium Used to Rear the Test Animal

In view of the fact that different experimenters are known to be using different media for rearing test animals and in view of the known influence of nutrition on susceptibility, a brief comparison was made of the susceptibility of *Drosophila* reared on banana, as used by the writer, and those reared on the potato–yeast medium suggested by Stultz (34). The tests were made with nicotine sulphate and the spray technique in the standard fashion. They were replicated five times. The results are shown in Table XIV.

The higher mortality in the check when the potato–yeast medium was used was due to a very high kill in the checks of two replicates. In still other tests replications were discarded because yeast, probably carried on the insect bodies, fermented the honey soaked plugs of vials in which flies were held previous to the mortality counts and resulting gases appeared to have anaesthetized the flies. Some difficulty was encountered in keeping available food plentiful in potato–yeast cultures and hence the flies of normal size. There are two factors involved, larval nutrition, which, if inadequate, results in undersized flies many of which may escape through the tulle covering containers, and adult nutrition, which we can only estimate from the mortalities, especially in the checks. Later it became necessary owing to a shortage in bananas, to use the potato–yeast medium to rear flies used in the tests recorded under in the following sections.

Variation Due to Different Experimenters

It is essential that a standardized toxicity test should give comparable results in the hands of different experimenters. To test this for the spray technique used, five workers were selected, the writer, a postgraduate student who had for some time previously assisted in this work, two fourth year students in entomology, and a girl studying the short course in household science and without previous laboratory training or experience. Nicotine sulphate was used in two concentrations (1.1% and 1.5% nicotine) and checks were tested with distilled water. Each experimenter tested each concentration on 10 containers of 15 flies each, each day. Eight replications were performed. The percentage mortalities obtained are recorded in Table XIII. The mortality data were transformed to the angular conversion values of Bliss's table and analysed by analysis of variance with the following results.

	Sum of squares	Degrees of freedom	Variance	*F*	*F* for 5% *P*	*F* for 1% *P*
Different workers	411.67	4	102.92	2.32	2.52	3.65
Different treatments	1100.68	1	1100.68	24.85	4.00	7.08
Different replications	1202.01	7	171.70	3.88	2.25	3.12
Interaction of workers and treatments	57.60	4	14.40			
Error	2790.89	63	44.29			
Total	5562.85	79				

It will be seen that the variance due to different workers, as shown in this test, might occur by chance in something more than 5% of such tests. There are probably some slight differences in mortalities as recorded by different workers but these are not as great as the differences due to different populations (replications) which are evidently significant. The interaction of workers and treatments is negligible. If the check cages, sprayed with water, are included as a treatment, to give another degree of freedom for treatments, and for the interaction, and 40 more for the total, the *F* value for treatment is greatly raised while that for workers becomes 2.31 and for replicates 3.66 indicating that the workers secured equally consistent results with treatments and check, while the check mortality varied less from population to population than did the mortalities with nicotine treatments. For this last reason it is deemed incorrect to include the water treatment in the analysis of variance as, compared to the nicotine treatments, it would not seem to be subject to equal chances of error.

Effect of Using Different-Sized Containers

The evidence secured by using different numbers of flies in a container, coupled with the two sizes of containers used when samples of 15 and 150 flies were compared, suggested a consideration of the possible effects of different-sized containers. To secure evidence on this point containers were prepared as in the earlier experiments but from glass tubing of five different inside diameters, viz., 9, 12, 14, 17, and 22 mm.

All containers were about 45 mm. long. Tests were then conducted using nicotine sulphate as a spray at two concentrations (1.1 and 1.5% nicotine) and a water check. Fifteen flies were used per container and all sizes were tested each day at each concentration. Eight replications were tested. The average percentage mortalities are given in Table XV. The following are the results of an analysis of variance performed on the angular mortality values.

	Sum of squares	Degrees of freedom	Variance	F	F for 5% P	F for 1% P
Different diameters	2841.79	4	710.45	16.35	2.52	3.65
Different replications	939.91	7	134.27	3.09	2.25	3.12
Different treatments	1003.02	1	1003.02	23.08	4.00	7.08
Interaction of diameters and treatments	320.32	4	80.08	1.84	2.52	3.65
Error	2737.73	63	43.46			
Total	7842.77	79				

It is evident that there is a significant difference in the observed mortalities obtained with different-sized containers. Inspection of the mortalities shows the greatest differences to occur between mortalities observed with the greatest and smallest diameters tested as compared with the three median sizes grouped together. The interaction of diameter and treatment is not significant but when we include the water treatment in our analysis this interaction reaches 2.99 (the F for 1% P for 60, and 8 degrees of freedom is 2.82). This is as we would expect since the check mortalities were unaffected by the container diameter. Again it seems that the water spray cannot be considered as a "treatment".

The causes of this variation resulting from container diameter remain to be investigated. The trend of the data suggests a parallelism between these and the results from lessening the number of flies in containers of constant size. Such an effect might be due to crowding (number of flies per unit of space), or to number of flies per unit of container surface area. Or it may be the effect of the diameter on the distribution and deposit of spray, as Potter (32) has shown such an effect when he varied the size of his spray tower.

This experiment is inadequate to finally determine the best container diameter to use. The best size, however, would seem to be that one by which day to day variation (due to population differences plus size and other factors) is least. An indication of the best of the sizes tested can thus be secured by treating each of the tests with each size as a separate experiment. Using the total mortalities for both concentrations for each day (expressed in angles) as normal variates, and determining the standard deviations for each, we arrive at the following:

Diameter of container, mm.	9	12	14	17	22
Standard deviation of results	1.97°	7.08°	3.83°	2.16°	2.21°

Within the limits of this experiment, which are recognized as very narrow, containers of a diameter of 9 mm. appear to give the most consistent results, while those of diameter 14 mm., used throughout all other tests, gave the second highest variation. It is proposed to further investigate this problem.

In general, it may be pointed out that all values obtained in toxicity tests to date, though functions of the type of insecticide and dosage, are not functions of these factors alone but are so definitely, also, functions of the test animal and technique used, that their value is purely relative and very limited. Thus a numerical *L.D.* 50 means nothing except in the light of the technique used. Only when one best technique, standardized in all its details, is used for all tests can results be compared accurately and even then the factors of different susceptibility of populations and of different test animals will remain.

Acknowledgments

As previously stated the data discussed in this paper have been accumulated since 1934 by a number of workers at Macdonald College, all under the direction of Dr. W. H. Brittain. Credit has been given for the various contributions in design of the apparatus, etc., made by Dr. J. McB. Cameron, Dr. M. L. Prebble, F. Cannon, and others, in the text and in the bibliography. The writer owes his gratitude to Dr. Brittain for continued assistance based on his invaluable experience; to Mr. Whitehead for the lettering on Illustration 13; and to Prof. Summerby, Macdonald College, and Dr. J. W. Hopkins, National Research Council, for help and suggestions with regard to the statistical analysis.

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Note: Tables will be found on pages 61 to 75.

TABLE Ia

DAILY DATA SHEET. INSECTS (*Drosophila*) KILLED BY A SPRAY TREATMENT WITH NICOTINE SULPHATE IN A 1% SAPONIN SOLUTION. ONE REPLICATION. MAY 17, 1938

Container	Water		Saponin, 1%		Nicotine, % conc.																																	
					0.01		0.05		0.10		0.15		0.20		0.30		0.35		0.40		0.45		0.50		0.55		0.60		0.70		0.80		0.95		1.10		1.25		1.40		2.00	
					Number of animals alive (*A*) and dead (*D*)																																				
	A	*D*	*A*	*D*	*A*	*D*	*A*	*D*	*A*	*D*	*A*	*D*	*A*	*D*	*A*	*D*	*A*	*D*	*A*	*D*	*A*	*D*	*A*	*D*	*A*	*D*	*A*	*D*	*A*	*D*	*A*	*D*	*A*	*D*	*A*	*D*	*A*	*D*
1	12	0	12	5	3	8	14	4	15	3	12	7	8	6	7	8	5	11	4	11	3	4	2	16	6	6	0	20	0	15	0	16	0	16	0	11	0	15	0	15	0	14
2	10	0	18	0	19	1	10	2	8	4	5	12	9	5	5	6	3	11	0	14	0	13	6	13	2	7	3	15	3	18	0	16	2	16	0	13	0	12	0	15	0	12
3	24	0	9	0	14	0	9	1	12	2	16	2	14	10	0	11	3	10	0	12	3	12	1	10	1	8	3	16	2	10	0	11	0	14	1	14	0	14	0	16	0	15
4	20	2	14	1	17	3	11	6	15	3	11	8	7	6	8	5	3	10	2	13	3	11	4	9	2	16	2	12	1	15	0	14	2	13	0	14	0	12	0	15	0	13
5	15	0	13	1	14	0	13	1	9	2	9	10	6	7	12	13	1	15	4	4	2	9	2	11	4	13	6	10	0	15	0	12	0	11	0	20	0	18	0	11	0	17
6	12	0	18	2	10	1	13	2	11	1	7	8	7	7	5	8	1	9	0	17	4	9	0	12	2	14	0	13	3	12	0	19	1	17	0	13	0	14	0	13	0	10
7	16	0	14	0	15	0	11	0	10	2	14	6	13	5	1	10	7	8	5	10	6	11	2	12	2	14	4	16	0	17	0	10	1	12	0	25	1	18	0	15	0	10
8	13	0	14	0	11	2	17	3	7	5	1	10	10	7	9	11	4	11	5	7	4	14	2	12	2	15	0	18	0	16	0	16	1	17	1	13	0	15	0	12	0	13
9	10	0	7	3	13	1	19	1	15	1	13	5	10	4	9	10	6	11	1	15	1	18	5	8	0	15	0	15	0	13	2	15	1	15	1	18	0	16	0	21	0	18
10	15	0	9	1	15	1	11	0	12	0	6	5	8	6	1	13	2	16	3	12	6	6	3	16	1	12	2	17	1	13	0	12	0	13	0	16	0	13	0	15	0	13
Totals	147	2	128	13	131	17	128	20	114	23	94	73	92	63	57	95	35	112	24	115	32	107	27	119	22	120	20	152	10	144	2	141	8	144	3	157	1	151	0	148	0	135
% Dead	1.34		9.22		11.49		13.51		16.79		43.71		39.87		62.51		76.26		82.73		76.98		81.51		84.51		88.37		91.56		98.60		94.74		98.13		99.34		100.00		100.00	

TABLE I*b*

INSECTS (*Drosophila melanogaster*) KILLED BY APPLICATION OF A SPRAY TREATMENT (1% NICOTINE SULPHATE IN 1% SAPONIN). SUMMARIZED DATA FROM ONE EXPERIMENT SHOWING AVERAGE PERCENTAGE MORTALITIES FOR EACH OF 10 REPLICATES

Date	Water	Saponin, 1%	Nicotine, % conc.																	
			0.01	0.05	0.10	0.15	0.20	0.30	0.35	0.40	0.45	0.50	0.55	0.60	0.70	0.80	0.95	1.10	1.25	1.40	2.00
			Percentage dead																		
May 11	0.0	4.1	5.2	8.5	13.0	22.8	35.3	51.7	62.4	62.4	66.0	69.7	85.5	77.8	83.3	92.7	95.8	98.7	98.0	100.0	100.0
May 14	0.0	5.3	8.7	9.2	18.2	32.4	29.3	64.8	79.6	75.6	77.6	78.4	90.6	94.1	93.6	97.1	97.6	95.7	98.8	100.0	100.0
May 17	1.3	9.2	11.5	13.5	16.8	43.7	39.9	62.5	76.3	82.7	77.0	81.5	84.5	88.4	91.6	98.6	94.7	98.1	99.3	100.0	100.0
May 19	0.7	6.3	5.2	6.1	14.5	20.3	41.3	60.9	70.9	66.1	80.6	75.5	87.6	86.0	94.5	95.1	97.8	97.1	98.7	100.0	97.6
May 21	0.0	6.0	5.2	3.3	18.0	13.8	24.0	57.8	60.9	59.3	70.0	65.4	74.8	79.1	87.6	91.3	95.0	95.2	95.6	99.3	100.0
May 26	0.0	2.7	1.2	1.2	8.5	21.2	27.0	37.9	54.1	51.8	62.3	72.0	74.0	79.8	72.8	91.1	93.7	92.5	90.8	99.4	100.0
May 28	0.0	2.7	2.5	6.6	13.0	17.4	22.2	41.3	45.4	49.0	47.0	53.6	61.2	71.1	74.5	86.2	95.4	97.4	95.3	99.5	100.0
May 31	1.2	8.1	10.2	13.0	19.1	24.0	32.7	59.3	65.9	69.9	70.0	70.2	87.3	89.0	83.3	89.1	93.4	99.5	99.5	98.4	100.0
June 2	1.0	4.5	2.1	4.9	17.3	11.1	25.0	34.9	44.2	63.1	64.1	66.3	79.1	71.4	70.0	73.7	89.8	95.1	98.7	97.1	98.8
June 4	0.0	4.7	5.5	11.2	11.9	20.0	40.0	39.0	58.5	47.0	62.2	61.5	75.0	71.0	79.4	87.9	89.6	88.9	96.8	95.4	96.2

TABLE Ic

AVERAGE LOG OF DOSAGE ($\bar{x}$), AVERAGE PROBIT ($\bar{y}$), SLOPE OF THE REGRESSION LINE (b), AND CHI² FOR EACH OF 10 REPLICATIONS USING NICOTINE SULPHATE IN A 1% SAPONIN SOLUTION AS SPRAY ON *Drosophila melanogaster*

Replicate	$\bar{x}$	$\bar{y}$	b	χ^2
1	1.62	5.47	3.019	26
2	1.58	5.68	2.955	89
3	1.59	5.73	2.835	31
4	1.60	5.64	3.265	23
5	1.64	5.44	3.213	21
6	1.68	5.44	3.138	49
7	1.69	5.32	3.341	58
8	1.64	5.60	3.057	49
9	1.69	5.40	3.066	50
10	1.69	5.44	2.550	36

TABLE Id

AVERAGE MORTALITY OF *Drosophila melanogaster*, OBTAINED FROM 4, 6, 8, AND 10 REPLICATES USING NICOTINE SULPHATE IN A 1% SAPONIN SOLUTION. ERRORS OF THESE EXPERIMENTS ARE SHOWN AS SECURED BY ANALYSIS OF VARIANCE AND ARE EXPRESSED IN ANGLES OF EQUAL INFORMATION.

	Nicotine, % conc.																		
	0.01	0.05	0.10	0.15	0.20	0.30	0.35	0.40	0.45	0.50	0.55	0.60	0.70	0.80	0.95	1.1	1.25	1.4	2.0
Four replicates																			
No. killed, %	7.37	9.34	15.56	29.80	36.44	62.45	72.25	71.70	82.79	76.05	87.03	86.52	90.75	98.37	96.25	97.39	98.70	100.00	99.39
Angles (average)	15.9	17.8	23.4	32.8	37.0	50.9	58.4	58.1	60.4	61.1	69.4	68.9	73.0	79.0	79.9	81.2	83.7	90.0	88.0
Standard error of a mean, 1.64°.																			
Variance:																			
Between replicates, 104.53.																			
Between treatments, 2267.09.																			
Error, 10.82.																			
Six replicates																			
No. killed, %	6.15	6.98	14.95	25.87	32.97	57.26	67.18	66.33	77.20	73.61	84.24	82.49	87.25	94.31	95.61	96.20	96.87	99.61	99.39
Angles (average)	13.7	14.5	22.7	30.1	34.8	48.6	55.4	54.8	58.4	59.4	66.1	66.9	70.1	76.8	78.7	79.4	81.0	88.1	89.0
Standard error of a mean, 1.02°.																			
Variance:																			
Between replicates, 277.05.																			
Between treatments, 3494.24.																			
Error, 6.29.																			
Eight replicates																			
No. killed, %	6.15	7.69	15.35	24.58	31.66	55.90	64.29	64.61	72.55	70.68	80.67	81.88	85.16	92.64	95.31	96.76	97.01	99.51	99.51
Angles (average)	13.8	15.4	22.9	29.3	34.0	47.7	53.6	53.8	56.3	57.5	64.6	66.2	68.3	74.0	78.0	80.6	81.6	87.6	89.0
Standard error of a mean, 1.21°.																			
Variance:																			
Between replicates, 274.57.																			
Between treatments, 4602.17.																			
Error, 11.73.																			
Ten replicates																			
No. killed, %	6.72	7.76	15.00	22.67	31.67	51.00	61.82	62.69	67.67	68.43	79.96	80.76	84.17	89.27	94.29	95.81	97.12	98.90	99.26
Angles (average)	13.29	15.56	22.76	28.03	34.08	45.63	52.03	52.58	55.56	56.65	63.97	64.44	66.56	72.87	76.73	79.42	81.72	85.76	87.47
Standard error of a mean, 1.45°.																			
Variance:																			
Between replicates, 335.48.																			
Between treatments, 5502.96.																			
Error, 21.09.																			

TABLE Ie

AVERAGE MORTALITIES (EXPRESSED AS PERCENTAGES) OBSERVED BY CAMERON AND PREBBLE (9) WHEN THEY SPRAYED ADULT *Drosophila melanogaster* WITH DIFFERENT CONCENTRATIONS OF NICOTINE SULPHATE IN 1% SAPONIN SOLUTION

	Nicotine, % conc.																		
	0.025	0.05	0.075	0.10	0.15	0.20	0.25	0.30	0.35	0.40	0.45	0.50	0.60	0.70	0.80	0.90	1.10	1.20	1.40
No. killed, %	4.85	8.61	16.3	17.1	23.3	38.1	34.3	40.5	51.7	62.0	63.8	72.3	78.0	79.9	90.2	92.8	94.9	96.1	96.6
Standard error	1.11	2.45	2.49	2.66	2.75	4.34	2.33	2.37	2.54	3.82	3.36	3.23	3.43	3.51	3.47	3.17	3.41	2.38	2.87
No. of containers tested	87	69	81	65	50	49	81	112	83	69	62	72	65	55	56	39	31	29	35

TABLE II

MORTALITY OF *Drosophila melanogaster*, OBTAINED WITH "NICOFUME" USED AS A SPRAY. (MORTALITY IS EXPRESSED IN PERCENTAGES AND ANGLES OF EQUAL INFORMATION. THE STANDARD ERROR, SECURED BY ANALYSIS OF VARIANCE, IS IN ANGLES OF EQUAL INFORMATION.) (10 REPLICATES)

	Nicotine, % conc.																		
	0.00	0.05	0.075	0.10	0.125	0.150	0.175	0.20	0.25	0.30	0.35	0.40	0.45	0.50	0.55	0.60	0.70	0.80	1.00
No. killed, %	0.45	2.24	4.85	12.68	18.73	25.29	34.61	34.70	50.94	57.78	68.32	79.87	87.03	89.96	94.11	96.40	98.30	99.99	99.94
Angle	—	8.1	11.86	20.13	25.92	30.55	36.54	36.13	45.33	49.47	55.98	63.47	69.64	72.47	76.68	79.64	83.52	87.29	89.43

Standard error of a mean, 1.25°.
Variance:
Between replicates, 137.15.
Between treatments, 7126.83.
Error, 15.72.

TABLE III*a*

MORTALITY OF *Drosophila melanogaster* OBTAINED BY THE SPRAY TECHNIQUE USING "NICOFUME" IN A 1% SAPONIN SOLUTION (10 REPLICATES)

	Nicotine, % conc.																			
	0.00	0.01	0.025	0.050	0.075	0.10	0.125	0.150	0.175	0.20	0.225	0.250	0.30	0.35	0.40	0.45	0.50	0.60	0.75	0.90
No. killed, %	0.61	3.67	4.75	4.32	7.74	9.73	10.58	19.45	23.59	28.20	33.90	43.75	48.89	52.25	64.02	60.09	77.50	87.14	92.76	98.06
Angle	—	10.03	11.67	11.43	14.55	17.48	18.52	26.03	28.90	31.65	33.66	40.15	43.87	47.91	53.38	—	62.81	69.83	75.43	82.90

Standard error of a mean, 1.33°.
Variance:
Between replicates, 489.16.
Between treatments, 6944.93.
Error, 17.70.

TABLE III*b*

AVERAGE MORTALITIES (EXPRESSED AS PERCENTAGES) OBSERVED BY CAMERON AND PREBBLE (9) WHEN THEY SPRAYED *Drosophila melanogaster* WITH DIFFERENT CONCENTRATIONS OF "NICOFUME" IN A 1% SAPONIN SOLUTION

	Nicotine, % conc.										
	0.01	0.025	0.05	0.075	0.10	0.125	0.150	0.20	0.25	0.30	0.40
No. killed, %	4.21	3.74	4.75	12.61	25.21	45.90	63.26	66.05	80.90	85.64	95.04
Standard error	± 3.16	1.00	3.60	1.40	4.06	0.53	2.99	3.97	3.97	3.18	3.59
No. of containers tested	82	92	175	176	177	210	251	90	85	85	92

TABLE IV*a*

MORTALITY OF *Drosophila melanogaster* OBTAINED BY THE IMMERSION TECHNIQUE USING NICOTINE SULPHATE (10 REPLICATES). (STANDARD CONTAINERS LOADED WITH 15 FLIES EACH WERE USED)

	Nicotine, % conc.																				
	0.00	0.20	0.25	0.30	0.40	0.50	0.60	0.70	0.80	0.90	1.0	1.1	1.2	1.3	1.4	1.6	1.8	2.0	2.5	3.0	4.0
No. killed, %	3.84	9.2	13.93	24.76	37.10	44.63	48.27	59.60	67.01	70.45	75.10	73.91	83.08	80.89	88.59	85.41	94.40	90.00	95.17	96.38	99.68
Angle	—	19.68	23.50	32.13	38.62	43.63	45.40	51.09	56.63	57.96	61.72	60.70	67.95	65.54	71.87	69.27	77.60	74.05	78.67	80.26	88.29

Standard error of a mean, 2.17°.
Variance:
Between replicates, 389.23.
Between treatments, 3734.60.
Error, 47.47.

TABLE IV*b*

MORTALITY OF *Drosophila melanogaster* OBTAINED USING NICOTINE SULPHATE BY THE IMMERSION TECHNIQUE AND TREATING CONTAINERS OF 150 FLIES EACH. (PERCENTAGE VALUES ARE GIVEN FOR EACH REPLICATE)

	Nicotine, % conc.																				
	0.0	0.20	0.25	0.30	0.40	0.50	0.60	0.70	0.80	0.90	1.0	1.1	1.2	1.3	1.4	1.6	1.8	2.0	2.5	3.0	4.0
No. killed, %																					
Repl. 1	1	6	7	9	7	15	18	30	52	50	62	66	80	71	75	91	75	62	96	98	100
Repl. 2	1	1	2	2	5	17	18	3	9	17	21	15	31	25	17	25	66	48	50	78	99
Repl. 3	1	6	3	1	4	10	10	10	11	17	16	19	13	19	33	64	70	62	74	66	92
Repl. 4	0	0	3	1	4	3	7	8	12	12	17	26	39	31	30	57	53	72	43	87	95
Repl. 5	0	0	1	1	7	12	5	10	15	5	16	26	20	21	32	37	66	25	58	84	98
% of total	0.6	3.35	3.65	2.65	5.18	11.77	11.48	11.20	23.28	17.39	25.18	30.20	33.24	31.52	39.57	53.54	58.76	58.00	61.76	84.48	97.17
Angle	—	6.82	8.10	8.54	13.30	18.76	19.36	19.28	25.22	25.40	30.16	32.86	36.70	34.72	37.42	48.42	54.42	47.16	54.68	66.70	81.18

Standard error of a mean, 3.13°.

TABLE V

MORTALITY OF *Drosophila melanogaster* OBTAINED BY THE IMMERSION TECHNIQUE USING NICOTINE SULPHATE IN 1% SAPONIN SOLUTION (10 REPLICATES)

	Nicotine, % conc.																
	0.00	0.10	0.20	0.25	0.30	0.35	0.40	0.50	0.60	0.70	0.80	0.95	1.25	1.50	2.00	3.00	4.00
No. killed, %	1.02	1.02	2.41	5.70	5.33	10.32	12.60	20.26	27.06	39.32	41.61	48.75	63.41	68.96	75.21	89.07	90.75
Angle	—	4.91	8.97	13.16	13.33	18.66	20.13	26.42	30.99	38.96	40.26	43.82	53.30	56.39	60.81	69.55	73.04

Standard error of a mean, 1.39°.
Variance:
Between replicates, 267.63.
Between treatments, 4869.31.
Error, 19·08.

TABLE VI*a*

MORTALITY OF *Drosophila melanogaster* OBTAINED BY THE IMMERSION TECHNIQUE USING "NICOFUME" (10 REPLICATES)

	Nicotine, % conc.																
	0.0	0.05	0.075	0.10	0.125	0.15	0.175	0.20	0.225	0.25	0.30	0.35	0.40	0.45	0.50	0.60	0.90
No. killed, %	3.17	3.79	9.12	12.09	17.14	22.43	35.83	34.62	45.13	48.34	71.49	60.98	83.03	87.70	88.33	96.61	99.56
Angle	—	15.03	19.77	22.44	25.85	29.57	35.24	37.52	42.84	45.26	58.46	52.96	67.05	70.65	70.90	80.55	87.15

Standard error of a mean, 1.86°.
Variance:
Between replicates, 224.25.
Between treatments, 5269.60.
Error, 35.20.

TABLE VI*b*

MORTALITY OF *Drosophila melanogaster* OBTAINED BY MORLEY (26) USING THE IMMERSION TECHNIQUE AND "NICOFUME"

	Nicotine, % conc.									
	0.0	0.2	0.3	0.4	0.5	0.6	0.7	0.8	1.0	1.5
No. killed, %	0.5	4.2	9.7	21.9	32.9	50.8	65.1	74.9	86.3	98.2
Standard error	—	0.03	0.11	0.45	0.70	1.34	1.62	2.2	1.4	0.85
Number of containers tested	70	60	70	70	70	50	50	40	50	30

TABLE VII*a*

MORTALITY OF *Drosophila melanogaster* OBTAINED BY THE IMMERSION TECHNIQUE USING "NICOFUME" IN A 1% SAPONIN SOLUTION (10 REPLICATES)

	Nicotine, % conc.															
	0.00	0.05	0.075	0.10	0.125	0.150	0.175	0.20	0.225	0.250	0.30	0.35	0.40	0.50	0.60	0.90
No. killed, %	3.67	1.49	4.26	9.26	17.86	26.29	37.16	46.49	48.12	53.84	66.96	76.96	83.76	90.87	98.32	100.00
Angle	—	12.91	15.78	20.54	26.98	32.79	39.16	44.16	44.91	48.52	56.46	61.69	67.91	73.89	84.04	90.00

Standard error of a mean, 1.60°.
Variance:
Between replicates, 90.97.
Between treatments, 5848.53.
Error, 24.78.

TABLE VII*b*

MORTALITY OF *Drosophila melanogaster* OBTAINED BY CAMERON AND PREBBLE (9) USING THE IMMERSION TECHNIQUE AND "NICOFUME" IN A 1% SAPONIN SOLUTION

	Nicotine, % conc.										
	0.01	0.025	0.05	0.075	0.10	0.125	0.15	0.20	0.25	0.30	0.40
No. killed, %	10.56	6.01	8.52	17.44	32.02	41.92	53.41	47.27	65.66	79.15	96.41
Standard error	6.33	0.98	8.04	2.50	3.18	2.39	3.18	2.48	2.33	2.72	0.90
Number of containers tested	91	92	32	32	32	92	92	91	92	89	88

TABLE VIII*a*

MORTALITY OBTAINED USING THE SPRAY TECHNIQUE AND NICOTINE SULPHATE IN A 1% SAPONIN SOLUTION ON FLIES (*Drosophila melanogaster*) OF DIFFERENT AGES (10 REPLICATES)

	Age of flies														
	1 day			2 days			3 days			4 days			5 days		
	Nicotine, % conc.														
	0.20	0.30	0.50	0.20	0.30	0.50	0.20	0.30	0.50	0.20	0.30	0.50	0.20	0.30	0.50
No. killed, %	3.92	10.08	19.07	3.56	6.27	16.54	3.04	3.85	14.66	2.72	3.81	12.15	4.04	6.03	18.22
Kill due to water, %	0.15			0.17			0.00			0.17			0.00		

TABLE VIII*b*

MORTALITY OBTAINED USING THE SPRAY TECHNIQUE AND NICOTINE SULPHATE ON FLIES (*Drosophila melanogaster*) OF DIFFERENT AGES (10 REPLICATES)

	Age of flies			
	1 day	2 days	3 days	4 days
	Nicotine, % conc.			
	1.1	1.1	1.1	1.1
No. killed, %	51.3	41.6	45.7	36.4

TABLE VIII*c*

MORTALITY OBTAINED BY CAMERON AND PREBBLE (9) USING NICOTINE SULPHATE AND 1% SAPONIN AS A SPRAY ON *Drosophila melanogaster* OF DIFFERENT AGES*

	Age of flies									
	Not over 3 days					Not over 4 days				
	Nicotine, % conc.									
	0.15	0.20	0.40	0.50	0.60	0.15	0.20	0.40	0.50	0.60
No. killed, %	7.7	20.5	51.3	64.7	68.2	23.3	38.1	62.0	72.3	78.0
Standard error	2.63	2.66	3.57	3.25	3.95	2.75	4.34	3.82	3.23	3.43
No. of containers treated	44	41	54	48	25	50	49	69	72	65

* *The percentage mortalities have been corrected by Abbott's formula, the corrections being based on the mortality obtained using 1% saponin alone.*

TABLE IX

MORTALITY OF *Drosophila melanogaster* OBTAINED BY THE SPRAY TECHNIQUE USING NICOTINE SULPHATE IN A 1% SAPONIN SOLUTION SPRAYED AT DIFFERENT RATES (EIGHT REPLICATES)

	Rate of spraying															
	1 cc. per 10 sec.								1 cc. per 25 sec.							
	Nicotine, % conc.															
	0.0	0.1	0.2	0.3	0.5	0.7	0.9	1.2	0.0	0.1	0.2	0.3	0.5	0.7	0.9	1.2
No. killed, %	1.62	6.96	16.8	21.1	42.4	58.6	68.2	76.1	1.47	6.26	14.5	22.7	43.4	51.0	61.1	74.5
Angle	—	14.3	22.7	27.3	39.4	48.4	54.2	60.4	—	13.5	21.2	27.6	41.1	45.2	49.9	59.9
	Standard error of a mean, 1.84°. Variance: Between replicates, 267.35. Between treatments, 2377.35. Error, 27.18.								Standard error of a mean, 1.61°. Variance: Between replicates, 222.26. Between treatments, 2224.98. Error, 20.72.							

TABLE X

MORTALITY OF *Drosophila melanogaster* OBTAINED BY THE SPRAY TECHNIQUE USING NICOTINE SULPHATE (10 REPLICATES)

	No. flies per container															
	15								150							
	Nicotine, % conc.															
	0.0	0.2	0.6	0.8	1.1	1.4	1.8	2.6	0.0	0.2	0.6	0.8	1.1	1.4	1.8	2.6
No. killed, %	0.85	9.44	31.40	39.70	56.60	63.80	77.70	88.80	1.81	28.15	64.8	79.8	88.6	91.6	96.8	98.8
Angle	—	16.28	35.05	40.70	48.88	54.74	62.51	72.32	—	30.47	52.58	63.17	70.09	73.76	80.04	84.95
	Standard error of a mean, 1.62°. Variance: Between replicates, 144.81. Between treatments, 3451.67. Error, 26.22.								Standard error of a mean, 2.24°. Variance: Between replicates, 141.78. Between treatments, 3461.29. Error, 49.59.							

TABLE XI

PERCENTAGE MORTALITY OF *Drosophila melanogaster* OBSERVED IN A COMPARISON OF NICOTINE SULPHATE WITH AND WITHOUT DIFFERENT CONCENTRATIONS OF SAPONIN, USING THE SPRAY TECHNIQUE (EIGHT REPLICATES)

	Nicotine, %								
	0			1.1			1.5		
	Saponin, %								
	0	1	3	0	1	3	0	1	3
Mortality, %	0.93	5.37	7.88	38.81	70.95	76.66	55.06	80.33	82.50
Angle	4.32	13.05	15.95	38.31	57.56	60.96	47.96	64.06	65.97

Variance:
Between replicates, 38.62.
For interaction saponin × nicotine, 80.50.
Error, 25.24.

TABLE XII

THE EFFECT OF THE NUMBER OF FLIES (*Drosophila melanogaster*) TESTED IN A CONTAINER ON THE PERCENTAGE MORTALITY OBSERVED, WHEN A NICOTINE SULPHATE SPRAY IS USED (EIGHT REPLICATES)

No. flies per container	Nicotine, % conc.		
	0	1.1	1.5
	Mortality, expressed as % dead		
5	0.76	55.5	70.4
10	1.21	68.4	78.6
15	0.36	73.5	79.3
30	0.97	80.2	90.6
75	2.05	83.3	87.4
150	3.22	82.7	93.5

TABLE XIII

PERCENTAGE MORTALITY OBTAINED BY DIFFERENT EXPERIMENTERS TESTING NICOTINE SULPHATE SPRAY ON ADULT *Drosophila* (FIVE REPLICATES)

	Experimenter				
	A	*B*	*C*	*D*	*E*
Mortality, % With water	0.4	0.6	0.1	0.4	0.2
With 1.1% nicotine	42.9	47.9	38.4	52.8	43.1
With 1.5% nicotine	56.1	57.2	56.2	66.0	52.9

TABLE XIV

PERCENTAGE MORTALITY OBTAINED TESTING FLIES (*Drosophila melanogaster*) REARED ON BANANAS AND THOSE REARED ON POTATO–YEAST MEDIUM WITH NICOTINE SULPHATE SPRAY (EIGHT REPLICATES)

	Treatment, nicotine conc. %		
	0	1.1	1.5
	Mortality, %		
Flies reared on banana	0.52	35.48	60.46
Flies reared on potato–yeast	3.59	46.74	61.24

TABLE XV

PERCENTAGE MORTALITY OBTAINED WITH NICOTINE SULPHATE SPRAY TESTED ON EQUAL NUMBERS OF FLIES (*Drosophila melanogaster*) IN DIFFERENT-SIZED CONTAINERS (EIGHT REPLICATES)

	Container diameter, mm.				
	9	12	14	17	22
Mortality, %					
With water	1.1	1.0	0.7	1.3	0.2
With 1.1% nicotine	64.5	55.2	51.4	51.2	28.1
With 1.5% nicotine	69.7	62.2	64.3	69.3	47.0

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